

Survival of *Clostridium difficile* spores on cotton during healthcare laundering

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Abstract

The transmission of *Clostridium difficile* infection (CDI) is mediated by spores, which are highly resistant to heat and disinfectants. The healthcare laundry policy, Health Technical Memorandum 01-04 Decontamination of linen for health and social care, provides minimum disinfection conditions and microbiological standards for laundered linen: no bacteria on previously sterile de-sized textiles, $>5 \log_{(10)}$ reduction of a thermotolerant species of bacteria and <100 cfu with no pathogenic bacteria on sampled linen. Quantification of the survival of spores, from hospital sheets (100% cotton) naturally contaminated with *C. difficile* spores were laundered in a washer extractor (WE) at a commercial laundry; they failed the microbiological standards. Similar results were achieved in a simulated healthcare WE cycle. The industrial detergent used failed the test for sporicidal activity (BS EN 13704), with a $2.81 \log_{(10)}$ reduction in spores. The method of recovering spores from swatches was important; in the presence of soiling, agitation by vortexing ($4.48 \log_{(10)}$ cfu/25cm²) was more effective than stomaching ($4.2 \log_{(10)}$ cfu/25cm², $p \leq 0.05$). Spore adherence to cotton occurred over time, with 0% (0 hours) and 51% (24 hours) adherence; adherence decreased to 34% (24 hours) after exosporium removal, suggesting a role in spore adherence to cotton. The possibility cannot be discounted that low-level spore survival on processed linen may be contributing to environmental contamination and asymptomatic carriage.

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Publications

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Abbreviations

AFM	Atomic force microscopy
AOB	Activated oxygen bleach
BHI	Brain heart infusion
BHIS	Brain heart infusion with supplements
BHIS/T	Brain heart infusion with supplements and taurocholate
CABA	Centre for disease control anaerobic blood agar
CACDI	Community-acquired <i>Clostridium difficile</i> Infection
CCFA	Cycloserine-cefoxitin fructose agar
CDAD	<i>Clostridium difficile</i> -associated diarrhoea
CDI	<i>Clostridium difficile</i> infection
CDRN	<i>Clostridium difficile</i> Ribotyping Network
CDMN	<i>Clostridium difficile</i> moxalactam norfloxacin agar
CFU	Colony forming unit
CMB	Cooked meat broth
CTW	Continuous tunnel washer
DoH	Department of Health
DPA	Dipicolinic acid
EDTA	Ethyldiaminetetraacetic acid
HCA	Healthcare assistant
HAI	Hospital-acquired infection
ICP	Infection control and prevention
ICU	Intensive care unit
MLVA	Multi Locus Variable-number tandem repeat analysis

MRD	Maximum recovery diluent
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
NHS	The National Health Service
NRES	National Research Ethics Service
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PHE	Public Health England
PMC	Pseudomembranous colitis
RFLA	Restriction fragment length analysis
SASPs	Small acid-soluble proteins
SE	Standard error of the mean
SEM	Scanning electron microscopy
TAED	Tetraacetythylenediamine
UK	United Kingdom
USA	United States of America
VRE	Vancomycin-resistant enterococci
WE	Washer extractor
WGS	Whole genome sequencing
WHO	World Health Organisation

1 Introduction

Healthcare institutions are facing a difficult challenge in the prevention and control of pathogens which are endemic in hospitals and healthcare facilities, with Infection Control and Prevention (ICP) teams being a vital part of the strategy to address rising nosocomial infection rates. Hospital Acquired Infections (HAIs) are not a new problem, and with CDI there are complex issues influencing transmission and aetiology (Khanna and Pardi, 2010; Knight *et al.*, 2010; Polage *et al.*, 2012; Kwon *et al.*, 2015).

A World Health Organisation (WHO) report of 2011 estimated that global rates of HAIs differ significantly based on economic resources. For example, high-income regions such as the UK, Canada and Japan ranged from 3.5 – 12% of patients acquiring HAIs, while across Europe the average was 7.1 % of patients with HAIs (World Health Organisation, 2011). Plowman *et al.* (2001) suggested that HAIs have an overall cost to society of around £3-11 billion in lost productivity. The latest figures show that around 1% of HAIs are caused by the endemic spore-forming bacteria *C. difficile* (Public Health England, 2016). The number of CDIs reported per month in England ranged from 321 – 467 in the September 2016 – September 2017 period (Public Health England, 2017).

First described in 1935, originally called *Bacillus difficilis* due to the difficulty in isolating the organism, *C. difficile* was eventually isolated from the intestinal flora of healthy neonates (Hall and O'Toole, 1935). The first recognised cases of *C. difficile*-associated infection (CDI) were later described in 1978 and it is one of the most common causes of nosocomial infectious diarrhoea (Larson *et al.*, 1978; Langley *et al.*, 2002; Cristina *et al.*, 2012; Richardson *et al.*, 2015).

The *Clostridium difficile* Ribotyping Network (CDRN) was set up in 2007 to assess ribotyped isolates for potential clusters, specifically where CDI incidence or severity seem to be increasing at the ward or hospital level. The hypervirulent 027 strain is thought to be responsible for a sharp rise in the incidence and an increased severity of infection from *C. difficile* to around 55,498 cases of CDI from April 2007 to March 2008 (McDonald *et al.*, 2005; Karas *et al.*, 2010; Khanna and Pardi, 2010). Public Health England records suggest that the number of *C. difficile* cases since 2007 have been in steady decline, dropping to 36,095 cases of CDI in 2008/2009. The decline started to slow in 2009/10, dropping to 26,504 cases of CDI, and 2010/11 to 21,707 cases. This trend continued in 2011/12, 2012/13 and 2013/2014 with 18,022, 14,694 and 13,362 cases of CDI, respectively. The decline in total cases of CDI came to a halt in 2014/15 and showed a small rise which continued in 2015/2016, with 14,192 and 14,139 cases of CDI respectively (Public Health England, 2016). In

England and Wales, there were 41, 500 mentions of CDI on death certificates for the period 2001-2013 (OFNS, 2013). Of the deaths during this period, 20,120 note *C. difficile* as the underlying cause of death.

C. difficile is a considerable burden to the NHS, Wilcox *et al.* (1996) found that patients stayed on average 21.3 extra days, which accounted for 94% of the extra cost. The total cost of contracting the infection totaled c. £4000 per hospital case (Wilcox *et al.*, 1996; Plowman *et al.*, 2001). Jones *et al.* (2013) standardised figures to 2010 prices, giving an updated cost of £6,986 extra per hospital case. With inflation, the costs per infection have almost certainly increased again, if prices have risen by 3/4 again, it could be in the region of £12,225 per hospital case in 2017.

The exact cost in financial terms to the NHS and other healthcare providers, will vary in different regions and countries, but all agree that CDI increases the cost in terms of extra care requirements, testing kits, staffing levels, longer hospital stays and *C. difficile* 'contract sanctions', where there has been any lapse in care identified (Wilcox *et al.*, 1996; Hansen *et al.*, 2007; Vonberg *et al.*, 2008; B. Mitchell and Gardner, 2012; Jones *et al.*, 2013; NHS England, 2016).

As well as financial concerns, there are the personal burdens carried by patients and their families. Patients have described their experiences regarding

feeling humiliated and embarrassed, lack of information about the disease, experiencing loneliness and anxiety when placed in isolation rooms and fear of recurrent episodes after treatment (Madeo and Boyack, 2010; Guilleman *et al.*, 2014).

Karas *et al.* (2010) suggested a global CDI attributable mortality level of 5.99% and noted the 2.5 fold increase in mortality after 2000, caused by the hypervirulent, NAP1/027 type. However, the authors also note that the number of deaths could also be influenced by better reporting of CDI. Mitchell and Gardner (2012) reviewed CDI mortality, including studies from 2005-2011, estimating a 5.7% to 6.9% CDI-attributable mortality rate, in support of the 5.99% suggested by Karas *et al.* (2010). The Mitchell and Gardner (2012) review included the later studies published 2009 to 2011. In North America, more recent figures suggest CDI-attributable mortality ranges from 5.7% to 16.7%, varying with endemic and epidemic periods (Kwon *et al.*, 2015). Recurrence of CDI is currently a major healthcare challenge and can increase mortality to ~30% (Evans and Safdar, 2015). Furthermore, ~30% of the patients with a first episode of CDI will experience a second episode and the probabilities to exhibit a third and fourth episode of CDI increase to 40% and 60%, respectively.

C. difficile is spread via the oral-faecal route and hypervirulent strains have been shown to spread between patients within hospital wards (Eyre *et al.*,

2013). In CDI strains which are not hypervirulent, there is evidence that CDI infections are not related to previous or current patients in a majority of cases and the source of these CDIs is yet to be established (Didelot *et al.*, 2012; Eyre *et al.*, 2013). One possibility to consider is inadequately decontaminated bed linens, which are processed at industrial laundries according to NHS laundry policy HTM 01-04 (Department of Health, 2016a). The minimum temperatures specified by the policy are 71°C for ≥ 3 minutes, or 65°C for ≥ 10 minutes (Department of Health, 2016a). In contrast to this, *C. difficile* has been shown to survive at 71°C for two hours and up to 90°C for 10 minutes (Rodriguez-Palacios *et al.*, 2010).

1.1 Epidemiology of *C. difficile* infection

C. difficile is an anaerobic bacterial species, which can colonise the intestinal tract of humans, mammals and other taxa (Songer and Anderson, 2006; Rupnik, 2007; Goorhuis *et al.*, 2007; Schoster *et al.*, 2012; Bailey *et al.*, 2016). Natural reservoirs can be found in domesticated livestock and *C. difficile* has been commonly cultured from animals which are slaughtered for human consumption (Schoster *et al.*, 2012). Some of the ribotypes previously isolated from animals are clinically relevant, such as ribotype 078 (Goorhuis *et al.*, 2007; Bailey *et al.*, 2016).

The *C. difficile* vegetative cell can form hardy endospores, which are the agent of transmission. Infection usually presents after a course of broad spectrum antibiotics, which reduce commensal species that regulate bile salts and leads to an over-abundance of cholates (Borriello, 1998; Giel *et al.*, 2010). These compounds then stimulate germination and outgrowth of *C. difficile* spores (Wilson *et al.*, 1982; Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2011). The main antibiotics associated with CDI are Clindamycin, Fluoroquinolones and Cephalosporins, although all classes of antibiotics have been linked to CDI, except aminoglycosides administered parenterally (Bignardi, 1998; Goudarzi *et al.*, 2014; Le Monnier *et al.*, 2014).

Other than antibiotic use, other risk factors which may predispose patients to CDI include increased age, duration of hospital stay, proton-pump inhibitor use and the severity of any underlying illness (Brown *et al.*, 1990; McFarland *et al.*, 1990a; Safdar and Maki, 2002; Lee and Cohen, 2013; Gerding and Lessa, 2015). There are other groups previously thought to not be at risk, who are now showing increased incidence, e.g. pregnant women, children and adults with no history of antimicrobial use (Garey *et al.*, 2008; Roupheal *et al.*, 2008; Gupta and Dubberke, 2014; Ye *et al.*, 2016).

The symptoms of CDI can range from minor self-limiting to watery fulminant diarrhoea and may result in Pseudomembranous Colitis (PMC) or

Toxic Megacolon (Doan *et al.*, 2012). Vegetative *C. difficile* cells produce two large toxins TcdA and TcdB, commonly referred to as toxin A (enterotoxin) and toxin B (cytotoxin) respectively, which cause severe inflammation and intestinal epithelial damage (Voth and Ballard, 2005; Bartlett, 2009; Navaneethan and Giannella, 2009; Carter *et al.*, 2012). The binary toxin is a virulence factor, which can be present in TcdA and TcdB negative isolates (McFarland *et al.*, 2007).

The course of infection is unpredictable and can become fatal in a short time when patients suffer co-morbidities (Kolli *et al.*, 2009; Mitchell and Gardner, 2012). In addition, asymptomatic carriage of *C. difficile* is seen in neonates and up to 3% of healthy asymptomatic adults carry *C. difficile* (Sherertz and Sarubbi, 1982; Kachrimanidou and Malislovas, 2011; Curry *et al.*, 2013). It has been estimated that in hospital settings and long-term care facilities asymptomatic carriage can be between 10% and 58% (Riggs *et al.*, 2007; Shaughnessy *et al.*, 2011). Asymptomatic carriage has been associated with protection from the most severe symptoms of the disease, but may proceed to symptomatic infection (McFarland *et al.*, 1990b; Furuya-Kanamori *et al.*, 2015).

Symptomatic CDI patients may shed 4 - 7 log₍₁₀₎ *C. difficile* spores/gram of faeces (Mulligan *et al.*, 1979; Al-Nassir *et al.*, 2008). In addition, asymptomatic patients have been shown to shed up to 5.9 log₍₁₀₎ *C. difficile* spores/gram faeces

(Riggs *et al.*, 2007). The infectious dose is unknown in humans, but believed to be very small at <10 spores; in animal studies, Larson *et al.* (1978) found that hamsters pre-treated with antibiotics developed disease when exposed to as little as 1-2 cfu (presumably spores) and untreated hamsters to resist infection even with exposure to $\sim 4 \log_{(10)}$ cfu. In addition, Lawley *et al.* (2010) developed a mouse CDI model, which found that exposure to 5 - 10 spores/cm² for 1 hour is enough for 50% of susceptible mice to become infected. The authors suggest this is likely to be a conservative estimate, as transmission occurred in a linear fashion based on exposure time. The most at risk groups in human cases are older patients with previous antibiotic use, who may also be hospitalised for longer periods, in which case, exposure times are likely to be much greater than 1 hour.

Given the generally accepted definition of a HAI, as infection acquired after 48 hours within a hospital environment, there are significant problems with defining true HAI and patients who have Community acquired *C. difficile* Infection (CA-CDI), which has been triggered by hospital admission followed by prescribed antibiotics.

1.1.1 Classification and biogeography

The standard method of classification of *C. difficile* in the UK is by ribotype, obtained by PCR amplification of the 16s-23s ribosomal inter-genic spacer

region, with visualization by capillary gel electrophoresis (Stubbs *et al.*, 1999; Xiao *et al.*, 2012). The *C. difficile* population structure has been studied using whole genome sequencing (WGS) showing it is clonal and distributed into six clades, which correlate well with multi-locus sequence types (MLST) and PCR ribotypes (Janezic and Rupnik, 2015). Toxigenic strains and strains exhibiting increased virulence are found throughout the clades. Five of the six clades (1-5) include toxigenic strains, and in clades 1, 4 and 5, toxigenic strains are combined with non-toxigenic strains (Table 1.1). The sixth clade (C-I) is associated with non-toxigenic strains only. Clade 1 is most heterogeneous in terms of STs (>100 STs) and PCR ribotypes. Well known PCR ribotypes present in clade 1 are 001 (ST-3), 012 (ST-54) and 014 (ST2, ST13, ST14, ST49, ST50, ST132). PCR ribotypes 014 and 001 are the first and second most prevalent strains in Europe, and the PCR ribotype 012 is the eighth most prevalent type in Europe. Clade 2 contains the epidemic ribotype 027 (ST1); in clade 3 ribotype 023 is most prevalent and is among the top 15 PCR ribotypes in Europe. Clade 4 is the A-B+ clade, represented largely by ribotype 017 (ST37), found in Asia and Europe. Clade 5 contains the epidemic ribotype 078 (ST11) and 5 other STs which are toxigenic and non-toxigenic. Whole genome comparisons have demonstrated that clade 5 is highly divergent from the rest of the population, separating about 1.1-85 million years ago. PCR ribotype 078 has just recently emerged as a human

problem, whereas previously it was most frequently associated with animals
(Janezic and Rupnik, 2015)

Table 1.1. Current grouping of selected *C. difficile* strains based on genomic comparisons (clades), MLST (STs) and PCR ribotyping and their correlations. (Reproduced from Janezic and Rupnik, 2015).

Clade	Number of STs	Prominent strains		Other PCR ribotypes represented within the STs
		PCR ribotype	MLST – ST	
1	104 ^a	RT001	ST3	RT009, RT055, RT072, RT077, RT115, RT262, RT268, RT305
	115 ^b	RT002	ST8, ST35, ST48, ST146	RT030, RT038, RT046, RT104, RT159
		RT012	ST54	-
		RT014	ST2, ST13, ST14, ST49, ST50, T132	RT007, RT020, RT025, RT076, RT095, RT129, RT169, RT220
		RT015	ST10, ST44	RT116, RT062
		RT018	ST17	RT052
2	19 ^b	RT027	ST1	RT016, RT036, RT176
	14 ^a	RT106	ST41, ST42, ST135	RT118, RT135, RT156, RT174, RT194, RT208, RT321
		RT244	ST41	RT156, RT208
3	4 ^a	RT023	ST5, ST22, ST25	RT063, RT212, RT122
	4 ^b			
4	13 ^a	RT017	ST37, ST86	RT407
	16 ^c			
5	3 ^b	RT078	ST11	RT033, RT045, RT066, RT126, RT127, RT193, RT237, RT280, RT281
	3 ^a			
C-I	5 ^d	ND	ST177, ST178, ST179, ST180, ST181	-

a= from Stabler *et al.*, (2012)

b= from Knetsch *et al.*, (2012)

c = from Griffiths *et al.*, (2010)

d= from Dingle *et al.*, (2014)

A European-wide surveillance group has been established which collects data regarding ribotypes and epidemic outbreaks; this has shown that three major European ribotypes have emerged 017, 027 and 078 (Dawson, Valiente *et al.* 2009). Bauer *et al.* (2011) reviewed the biogeography of ribotypes across sites in Western Europe, with results suggesting each country has a unique community of ribotypes present. Vaneck *et al.* (2012) studied ribotype frequency in North-East England and found the five most numerous ribotypes were 001 (15.6%), 106 (11.7%), 027 (10.6%), 015 (7.7%) and 078 (5.6%). The ribotype distribution changed over time; from July 2009 to December 2010, the proportions of ribotypes 002 and 015 decreased and the proportions of ribotypes 016 and 023 were found to increase.

Due to enhanced fingerprinting by multi locus variable-number tandem repeat analysis (MLVA) the relatedness of strains can be resolved with more accuracy when suspected clusters belong to the same ribotype. In a study by Public Health England (2011), 19% of potential outbreaks of CDIs, which belonged to the same ribotype, actually comprised of unrelated isolates as shown by MLVA. These findings emphasise the value of enhanced fingerprinting to confirm or refute suspected CDI case clusters. MLVA is likely to be phased out and replaced by whole genome sequencing WGS according to Public Health England (Public Health England, 2016). This WGS technology provides very similar results regarding identifying clusters, although it focuses on a greater

number of different gene regions, while also providing antibiotic susceptibility and virulence gene data (Public Health England, 2016).

1.1.2 Overview of potential transmission routes

In recent years important potential sources of *C. difficile* infection have been considered, including surface contamination within the hospital, health care worker (HCW) hands, asymptomatic carriers, aerial dissemination and fabric items within the ward. These were considered and compared to understand potential transmission routes and sources of infection.

1.1.2.1 Links between surface contamination and CDI rates

The emerging patterns of *C. difficile* environmental contamination suggest spores are more likely to be recovered from a room currently housing a CDAD patient, than a room with a non-CDAD patient (Dubberke *et al.*, 2007). *C. difficile* spores are frequently disseminated within the near patient area, particularly high touch areas, and more widely to other areas of the ward (Eckstein *et al.*, 2007; Best *et al.*, 2010). Within the near patient area, the 'dirty areas' such as toilets, toilet floors and commodes are more often positive for *C. difficile* spores than 'clean areas' such as telephones and bed tables (Dubberke *et al.*, 2007). In addition, Vonberg *et al.* (2008) found heavy spore contamination on bed frames, commodes, floors and bed pans. Interestingly, Dumford *et al.* (2009) found that *C. difficile* spores were also isolated in rooms

of patients not infected or colonised with *C. difficile*, as well as areas not associated with patients such as nurse/physician work areas on telephones and keyboards. Evidence of spores in non-CDI areas suggests that spores may be introduced or transported to other areas such as nurse stations, perhaps by hand carriage or portable equipment (Dumford *et al.*, 2009).

There is a link between spore contamination of surfaces and subsequent transmission, this has been shown where cleaning related interventions have coincided with reduced CDI rates (Kaatz *et al.*, 1988; Mayfield *et al.*, 2000; Wilcox, 2003; Boyce, 2007; Dubberke *et al.*, 2007; Weber *et al.*, 2013; Alfa *et al.*, 2015). Interestingly, there have also been cases where no reduction has been seen after intervention or conflicting results, which highlight the limited understanding of the role of surface contamination in CDI rates (Wilcox, 2003; Pepin *et al.*, 2004).

There are various methods of intervention which provide evidence of the link between surface contamination and CDI rates, these are disinfection product substitutions, cleaning regime changes and whole-room automated disinfection using hydrogen peroxide fogging (Donskey, 2010). Most, but not all have included sampling to check for spore contamination of surfaces as a measure of the efficacy of the intervention. Kaatz *et al.* (1988) found unbuffered hypochlorite (1:10) reduced surface contamination with spores to 21% of the

previous load and ended an outbreak in the study ward. In addition, a single room was disinfected with phosphate buffered hypochlorite, which reduced surface contamination with spores by 98%. The study provided early evidence that spores were becoming endemic in hospital wards, but could be eliminated with a sporicidal agent and the number of CDIs could be reduced. This early work did not consider other factors such as antibiotic usage over the study period, which may also have reduced CDI rates. Mayfield *et al.* (2000) supported the findings of Kaatz *et al.* (1988) when assessing the effect of unbuffered hypochlorite (1:10), on the overall rate of CDI in three hospital wards. On the first ward CDI rates were reduced from 8.6 cases/1000 patient days to 3.3 cases/1000 patient-days, but unfortunately, there was no monitoring of the levels of surface contamination. In the second and third wards, the effect was not reproduced; there was no reduction from their baselines of 3 and 1.3 cases/1000 patient days, respectively. The first high incidence ward benefited from the enhanced cleaning and the rate of CDI was reduced to a similar level as the other wards studied, but there was a limit to the effectiveness. There was no consideration for any other factors that may have affected the intervention, such as changes in antibiotic usage and the method of the cleaning i.e. detergent use to remove visible soil before disinfecting or reduced cleaning compliance.

You *et al.* (2014) found a combination of staff education, patient isolation measures and cleaning twice daily with sodium hypochlorite (1000ppm), significantly reduced *C. difficile* infection rates by 67%. This suite of measures implemented on a single study ward reduced CDI cases from 4.70 to 1.53 cases/1000 patient-days. In contrast, staff education and isolation measures alone were performed across the rest of the study site and CDI rates were seen to increase, from 1.09 to 1.47 cases/1000 patient-days although this was not statistically significant. The reduction of CDI rate in the sodium hypochlorite intervention ward provides a strong link between environmental contamination and CDI rate. The authors note that antibiotic prescribing remained the same, but hand hygiene compliance significantly increased during the study period. The increased hand hygiene compliance may have affected CDI rates, due to reduced potential hand transmission. No environmental samples were taken and compliance with cleaning protocols was not assessed. These data would give more robust evidence of the decreased CDI rate being directly attributable due to a reduction in *C. difficile* spores, assuming spore contamination was shown to be reduced.

More recently, disinfectant wipes with sporicidal action were shown to significantly reduce CDI rates when combined with monitoring of cleaning. Alfa *et al.* (2015) demonstrated that when disinfectant cleaning wipes (with a sporicidal claim) were applied daily to patient care high-touch environmental

surfaces, with a minimum of 80% compliance, the rates of CDI were significantly reduced. The previous 52-week period rate was 5.6/1,000 patient days, which reduced to 3.9/1,000 patient days after the intervention.

In contrast to the studies outlined, others have found conflicting evidence and no reduction in CDI rates with the use of different products or cleaning schedules. For example, Wilcox *et al.* (2003) assessed the effectiveness of using a chlorine-based disinfectant compared to a neutral detergent in a cross-over study in two hospital wards. The disinfectant or detergent was used alternately on each ward for 6-12 months over 2 years. Both hospital wards, X and Y, had similar numbers of positive surface samples for *C. difficile* spores during the study, but on X ward there was a statistically significant reduction in the rate of CDI from 8.9 to 5.3 cases per 100 admissions, during use of the hypochlorite cleaner. The reduced rate was equivalent to 17 fewer cases during the hypochlorite period. In Y ward, there was an increase in CDIs during hypochlorite use from 3.5 to 4.7 per 100 admissions, although it was not statistically significant. No changes in antibiotic prescribing were noted on either ward. These markedly different results, on two very similar hospital wards, highlight a poor understanding of confounding factors when measuring CDI risks and surface contamination.

Valiquette *et al.* (2007) found no difference in the rate of CDI when strengthening the cleaning protocol by adding a sodium hypochlorite step for terminal cleaning of CDI rooms and ward disinfection where there were more than 3 cases of CDI. The disinfection agent was later changed to 7% activated hypochlorite, which again made no difference to the CDI rate. In addition to the change in disinfection protocol, staff education on isolation measures and the importance of cleaning were frequently repeated. Ultimately, the only intervention that affected CDI rate, in this case, was voluntary restriction/altered prescribing after widespread education about the classes of antibiotics associated with CDI. Unfortunately, there were no surface samples taken, nor monitoring of cleaning, so there was no measure of whether cleaning had been effective at reducing surface contamination.

Finally, Khanafer *et al.* (2015) reviewed the effects of interventions on CDI rates in 21 studies. Due to difficulties in how the studies were designed and reported, there were few evidence-based recommendations. However, broadly speaking the authors suggest the best interventions were to reduce the number of susceptible patients by reducing antibiotic use, followed by using gloves and washing hands, and daily cleaning of hard surfaces with a bleach-based product.

In summary, the evidence is somewhat undermined by the lack of monitoring, concomitant interventions and other unintended interventions,

such as unexplained compliance in hand hygiene or changes to antibiotic prescribing during study periods. There is evidence that effective cleaning practices, such as using hypochlorite-based products, contribute to a reduction in surface contamination and often, but not always result in reduced CDI rates. There appears to be a minimum baseline rate of around 3 cases/1000 patient days, beyond which cleaning has no effect.

1.1.2.2 Hand carriage of *C. difficile* spores by Health Care Worker

Surface contamination with *C. difficile* spores can contribute to Health Care Worker (HCW) hand carriage, as can direct contact with a patient's skin or faecal soiling (McFarland *et al.*, 1990b; Riggs *et al.*, 2007). HCW hand carriage is considered a significant factor in lateral transmission between patients (Bobulsky *et al.*, 2008; Cohen *et al.*, 2010; Jullian-Desayes *et al.*, 2017; McFarland *et al.*, 1990a). For example, an early estimate found caring for patients with CDI can lead to significant HCW hand carriage of up to 59%; although this was a measure of total *C. difficile* with no differentiation between the number of vegetative cells and spores in the protocol (McFarland *et al.*, 1990b). Given that vegetative cells can survive in moist aerobic environments for up to 6 hours, total spore carriage is not clear (Jump *et al.*, 2007). Later estimates found 29% and 14% hand carriage of *C. difficile* after patient care activities (Struelens *et al.*, 1991; Samore *et al.*, 1996).

Samore *et al.* (1996) reported a 14% hand carriage rate for *C. difficile* but samples were taken within 30 minutes of leaving the patient isolation room. The time period between care and sampling might explain the lower proportion of hand contamination in this study, as spores may have been removed by hand washing or transferred to other surfaces.

Landelle *et al.* (2014) specifically quantified *C. difficile* spore hand carriage after CDI patient care and found 24% positive hand carriage, in HCWs exposed to CDI patients and compared to 0% in control group HCWs who worked in non-CDI wards. Positive hand carriage of spores was associated with high risk activities i.e. helping with washing or contact with faecal soiling, and with not wearing gloves during care activities. In addition, a recent update found levels of HCW hand carriage of *C. difficile* spores to be relatively low, at 1.4% (1/71) (Sasahara *et al.*, 2016). HCWs were screened for *Bacillus* spp. and *C. difficile* spores, although 76.1% of HCWs' hands tested positive for spores, these were almost exclusively *Bacillus* spp. and just a single sample positive for *C. difficile* spores, with 50 cfu/hand. The overall *C. difficile* spore load was low compared to hand carriage of *Bacillus* spp. where the mean was 468 cfu/hand. The HCWs were caring for a single CDI patient, so this could explain why the *C. difficile* spore hand carriage rate was low (Sasahara *et al.*, 2016). In situations with more CDI patients, there could be a higher rate of hand carriage.

Alternatively, hand hygiene compliance may be improving, but it is difficult to know without specific compliance monitoring.

Furthermore, Mutters *et al.* (2009) found no significant difference in *C. difficile* contamination on HCW hands when working in CDI and non-CDI wards, with counts of 1549.7 and 215.6 by real-time polymerase chain reaction (PCR), respectively. These high levels of hand carriage were not significantly different, despite the CDI wards having higher numbers of positive environmental samples. Care should be taken with comparing this study to the previous examples as the level of *C. difficile* was quantified using real-time PCR and not an enumeration of viable spores. Real-time PCR would have included vegetative cells/spores which were present but not viable. Significant correlations were observed between *C. difficile* levels on the floor and the hands of patients and HCWs' hands, in ward areas without evidence of *C. difficile*. The *C. difficile* counts on HCWs' hands were also highly associated with the positive environmental samples in the near-patient area and distant areas, which suggests the HCWs' hands were important in the spread of *C. difficile* spores within the hospital environment and between patients (Mutters *et al.*, 2009).

A recent review of the literature on the transmission risk of hand carriage of *C. difficile* spores, which included the previously discussed studies concluded HCWs hands are a likely source of *C. difficile* spores (Jullian-Desayes *et al.*,

2017). The authors also note the differences in sampling techniques and time between patient contact and sampling.

In hospital wards without CDI diagnosed patients, caring for asymptomatic patients can also lead to significant hand carriage of *C. difficile* spores in HCWs (Riggs *et al.*, 2007). This is likely due to the high number of spores which are shed (up to 5.9 log₍₁₀₎ cfu/gram faeces), skin contamination and the potential lack of isolation measures, such as not using gloves, perhaps due to a perceived lower risk.

1.1.2.3 Asymptomatic carriers

In institutions where CDI incidence has been reduced by infection control regimens directed at symptomatic CDI patients, asymptomatic carriers have been shown to be a potential source of infection. For example, Curry *et al.* (2013) found that a quarter of hospital-acquired CDI cases in their study had isolates which were highly related to isolates from patients identified as asymptomatic carriers. Staff have been considered as potential asymptomatic carriers, but Hell *et al.* (2011) found that healthy HCWs faecal tests were all negative for *C. difficile*. Their aim was to quantify the asymptomatic carriage and shedding of spores in HCWs and relate this to the potential risk of transmission to vulnerable patients. The control was a group of administration workers who also tested negative. The study involved a small number of participants and would need further sampling in multiple regions to be confident of these

findings. What the result does suggest is that estimates of 3%-10% asymptomatic carriage, in the community and particularly in HCWs, are potentially overestimated (Kachrimanidou and Malisiovas, 2011; Didelot *et al.*, 2012). In the long-term care environment asymptomatic carriage has been increasing, estimates range from 21%-51% (Riggs *et al.*, 2007; Rea *et al.*, 2012; Ponnada *et al.*, 2017).

1.1.2.4 Aerial dissemination

Early investigation of CDI transmission by aerial dissemination found no evidence of *C. difficile* spores in air samples (Larson *et al.*, 1980; Toshniwal *et al.*, 1981). Due to the short sampling times and the limited number of air samples taken, the nature and extent of the aerial dissemination were not understood. Toshniwal *et al.* (1981) studied the effect of conventional and 'clean' rooms on hamster mortality after Clindamycin treatment as a model for human CDI. There were ten air samples of 20 ft³ air over 30 minutes and all were negative for *C. difficile* spores. Details of when air samples were taken were not stated, such as concurrent activities e.g. feeding or cage cleaning. There is now evidence suggesting aerial dissemination of *C. difficile* spores is common but sporadic, which could explain the lack of positive samples in earlier studies. Roberts *et al.* (2008) found preliminary evidence of aerial dissemination of *C. difficile* spores, during two air sampling sessions in a six-bedded elderly care bay. There were 23 positive samples from the first session; mean counts were 53-426 cfu/m³ of

air. Ribotyping found 22/23 positive samples to be ribotype 001. There were no positive samples from the second air sampling session, supporting a sporadic nature to the aerial dissemination of spores.

Best *et al.* (2010) later confirmed aerial dissemination of *C. difficile* spores, 7/10 intensively studied symptomatic CDI patients had room air samples that were positive for *C. difficile* spores (1-2 colonies). The air was sampled for 10 hours over 2 days, as well as environmental sampling around the patient and in more distant areas. Environmental samples were positive for 9/10 patients. Overall, 60% of patients were positive for *C. difficile* spores in both room and air samples. The number of colonies was shown to increase after activities such as the lunch period or visitors. However, the increase was marginal, with combined results showing an increase from 1 colony recovered at times of low activity (no visitors or rounds) to 8 colonies recovered at the peak of activity during the lunch and visiting time. In addition, because all air, environmental and patient samples were analysed by ribotyping and MLVA, the data provide evidence of the link between the patients' CDI strain, environmental contamination and aerial dissemination. The authors suggest that the patients and surfaces were contributing to the aerial contamination, but the reverse could also be true when the spores eventually settle out from the room air. Aerial dissemination may explain why *C. difficile* spores can be found on low-touch areas which are physically located high up in patient rooms (Shiomori *et al.*, 2002).

1.1.2.5 Textiles as sources of CDI

Textiles in hospitals are already considered to be a potential route of HAIs and so their importance must be considered in regard to *C. difficile* spores (Sattar *et al.*, 2001; Shiomori *et al.*, 2002; Fijan *et al.*, 2008; Weiner-well *et al.*, 2011; A. Mitchell *et al.*, 2015; Bockmühl, 2017).

Perry *et al.* (2001) found 22/57 (39%) uniforms to be contaminated with at least one pathogenic organism (Methicillin-resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Escherichia coli* (VRE) and *C. difficile*) at the start of duty. Of those positive samples, 7/22 were positive for *C. difficile* spores on entering the ward, conversely at the end of the shift 11 were positive for *C. difficile* spores. All uniforms which tested positive for *C. difficile* had between 1-10 cfu. The study highlighted other concerning behaviour, such as washing uniforms at a maximum of 50°C (due to fabric labels or longevity of the garment) and washing with other items of clothing on water-saving cycles. Washing in a general load could lead to cross-contamination while over-loading the machine may increase the fabric to water ratio preventing adequate dilution and removal of the pathogenic organisms.

Patel *et al.* (2006) found that tumble-drying and ironing were adequate to render home-laundered uniforms free from *Staphylococcus aureus* and environmental bacteria picked up during the wash cycle. However, the methods used in this study were not applied to *C. difficile* contamination of uniforms.

White coats have been shown to be particularly susceptible to transmission of hospital pathogens such as MRSA (Treakle *et al.*, 2009).

Additionally, MRSA has been associated with airborne and surface contamination related to dissemination of bacterial cells during bed-making (Shiomori *et al.*, 2002). In a study by Shiomori *et al.* (2002), bed-making increased the mean number of MRSA sampled from the air from 4.7 to 116 cfu/m³ of air, a 25 fold increase in the number of viable airborne CFUs. After bed-making, the number of CFUs on bed sheets was almost undetectable. In the 30 and 60 minute samples after bed-making, the number of MRSA sampled from the air was not significantly different from the resting period. The authors suggest that the presence of bacteria on sheets can contribute to surface contamination, through their release during bed-making. Further work is needed to understand the extent to which viable bacteria or spores on sheets can contribute to surface contamination during bed-making.

1.1.2.6 Body of evidence of transmission within hospital environments

There is reasonable evidence for surface contamination and increased rates of CDI, contribution of HCW hands in the transfer of spores to distant areas and aerial dissemination of spores. However, there is surprisingly little tracking or evidence of transmission from one patient to another, outside outbreak case studies. There is little doubt that the hypervirulent strain NAP1/027 caused significant mortality and morbidity among inpatients *circa.* 2007 (O'Connor *et*

al., 2009); since then there have been several studies looking at transmission, patient contacts and relatedness of isolates to identify other sources of infection in non-hypervirulent cases.

Samore *et al.* (1996) found a single hypervirulent strain (named D1 by the authors) of *C. difficile* was associated with high surface contamination, HCW hand carriage and transmission to contacts shown by indistinguishable isolates from index and contact cases. The method of comparing molecular relatedness was by Pulsed-field gel electrophoresis (PFGE) or by restriction fragment length analysis (RFLA) which are not as sensitive as MLVA at establishing relatedness (Public Health England, 2016). The relatedness between index and contact isolates is inferred, but with better discrimination a higher number of unrelated isolates might have been exposed. In contrast, most episodes of CDI that were due to all the other strains sampled (not D1), were not associated with transmission to contacts and never with significant clusters. Overall, 14/25 cases had no known source of infection. The source of the other strains in this study is unknown. The authors suggest an unknown environmental reservoir, such as asymptomatic patients, but this is speculation and not within the scope of the work.

Similarly, Shaughnessy *et al.* (2011) found unexplained cases when investigating prior room occupants' CDI status as a risk factor for CDI in a

hospitalised patient. The study was conducted on an ICU ward, as single rooms were conveniently available. Of the 1770 patients admitted to the ICU over the study period, 77/1679 (4.6%) acquired CDI in a side room without the previous occupant having CDI. In contrast 10/91 (11%) acquired CDI in a room where the previous occupant had CDI. In short, the likelihood of acquiring CDI was significantly higher in rooms where the previous occupant had CDI. This remained significant after controlling for other accepted risk factors such as age, proton-pump inhibitor use and antibiotic use. Once again, this study was limited as patient isolates were not ribotyped or analysed by MLVA to evaluate transmission from the previous occupant. In addition, there were no environmental samples taken to detect persistence of *C. difficile* spores within the room. Without knowing if there was any environmental contamination or the ribotype of the prior or current patients' strains, transmission can only be inferred. If we accept the inference of direct transmission, only the CDI status of the patient immediately before the current patient was considered as a risk factor. In addition, only development of CDI at the time of discharge from the ICU was considered as a possible transmission event, whereas community-onset of a HAI could present post-discharge. Accessing data for subsequent development of CDI after transfer from ICU to other hospital wards, combined with MLVA of isolates would have provided a bigger sample with which to assess the transmission risk.

Echaiz *et al.* (2014), investigated the risk of contracting CDI during co-occupancy alongside a roommate with diagnosed CDI. Two groups were defined, patients who shared a room with a CDI patient and those who shared a room with a non-CDI patient. The overall finding was that 9/120 (7.5%) exposed roommates developed CDI and 4/125 (3.2%) non-exposed roommates developed CDI. The difference was not statistically significant and the author could only suggest a trend toward increased risk when a co-occupant had diagnosed CDI. The sample size in this study was a major limitation to understanding the risk of co-occupancy in CDI transmission, as was the lack of ribotyping to assess the relatedness of isolates. The transmission events were again merely inferred, as there was no direct evidence of relatedness between the original CDI isolate and the subsequent CDI in the roommate.

In contrast, Didelot *et al.* (2012), used WGS to assess just such relatedness between past and present cases and found that direct transmission from one room occupant to another is much less common than previously thought. The authors constructed a picture of the relatedness of isolates collected in Oxfordshire Trust hospitals over a four year period and matched the cases to potential patient links. By comparing isolates pairs with the same Sequence Type (ST), but separated by 1-561 days between isolation, a short-term molecular clock was calculated. The molecular clock and patient information were used to identify related isolates; there were only 67/358

isolate pairs with time to most recent common ancestor of less than six months in all sequence types except ST1 (which corresponds to ribotype 027, hypervirulent strain).

Within ST1, the rate was 167/267, which is consistent with the hypervirulent nature of ST1 strains. In short, only 18% of non-hypervirulent cases were closely related enough to be considered a direct transmission event by the authors' criteria. In contrast, 62.5% of hypervirulent cases were closely related enough to be considered a direct transmission event. Therefore, the source of infection for a significant majority (82%) of the non-hypervirulent infections, and over a third (37.5%) of hypervirulent cases, unaccounted for by the measures employed. Therefore, there must have been other sources involved in transmission, besides the patient contacts identified in these cases.

Eyre *et al.* (2013) went further and used MLVA and WGS to assess the relatedness of all *C. difficile* positive samples in Oxfordshire, over a three year period. A rate of within-host diversity and evolution was generated by comparing the genetic sequence of the first and last samples taken from a subset of 145 patients (where the sequence type was the same). The result was an estimate of 0-2 single nucleotide variants (SNV) between isolates sequenced 0-124 days apart and 0-3 SNV between isolates sequenced up to 364 days apart. Using this rate all samples processed during the study were assessed for

relatedness and potential ward, hospital or other physical contact. Of the 957 sequenced isolates, 333 (35%) were related to at least one other sequenced isolate, with <2 SNVs; these were considered potential transmission events and hospital contact was assessed. Overall, 126 cases had full ward contact, 5 on the same ward but after discharge/recovery of a potential contact (>28 days apart), 29 were in the same hospital on different wards and 21 were linked by the hospital and were on the same ward within 28 days.

There were 152 cases, related to at least one other case, for which no hospital-based link was found, although 32 had either the same General Practice or lived within the same postcode. The remaining 120 cases had no record of hospital/community contact to the genetically related case. The authors discounted potential laboratory contamination, as the isolates were processed in weekly batches and the median time between related cases was 113 days. Conversely, the other 624 (65%) isolates sequenced had >2 SNVs which by the criteria of this study, were genetically distinct from all other isolates in the study. In short, there was no transmission from symptomatic patients in nearly 2/3 cases. In addition, 45% of all cases had >10 SNVs which indicates a variety of distinct sources. The authors speculate that most CDIs were acquired from asymptomatic carriers or an unknown environmental reservoir.

In conclusion, the baseline number of apparently unrelated *C. difficile* infections occurring in healthcare settings, are from currently unknown sources. It continues to be the case that length of hospital stay and even the CDI rate of hospitals are linked to individual patient CDI risk (Miller *et al.*, 2016). Therefore, consideration of other sources within the hospital environment that may contribute to the transmission of CDI is warranted. One such transmission route that has yet to be fully explored is fouled and infected linen, and the implications of the decontamination requirement of commercial laundering.

1.2 Healthcare laundry

1.2.1 Healthcare laundry policy for soiled/infected bed linen

The main purpose of laundering hospital bed sheets is to provide clean and safe bedding for use on all hospital beds. Given the vulnerable status of the general hospital population, comfort and freedom from contamination are paramount concerns. Regarding health and safety at work, there is also a cause for concern, in terms of laundry operative safety, when sheets are handled throughout the laundry process. Commercial laundries can be accredited to the British standard BSEN 14065:2016 Laundry processed textiles. Biocontamination control system (British Standards Institution, 2016). Accreditation demonstrates that the

laundry system meets the basic requirements of biocontamination control when processing linen from health and social care institutions.

The health and social care linen policy which covers handling and processing has been developed with a greater emphasis on an evidence-based approach (Department of Health, 1995; Department of Health, 2013; Department of Health, 2016a). The bed linen from CDI patients was previously handled and laundered according to the NHS policy 'HSG (95) 18: Hospital Laundry Arrangements for Used and Infected Linen' (Department of Health, 1995). The policy was updated in 2013 to become the Choice Framework for Local Policy and Procedures 01-04 – Decontamination of linen for health and social care (CFPP01-04). The latest edition was released in 2016, called Health Technical Memorandum 01-04: Decontamination of linen for health and social care. The latest policy HTM 01-04, sets out the same conditions as the previous iterations, regarding thermal and chemical disinfection, mixing time and water volume to fabric ratios. The HSG (95) 18 policy, had no specific mention of the possible presence of *C. difficile* spores (Department of Health, 1995), in the CFPP 01-04 update in 2013, the first mention of *C. difficile* spores alluded to the potential risk they posed, but no references were given regarding the evidence base for this comment (Department of Health, 2013). The HTM 01-04 update has the addition of an information box which states that a small number of *C. difficile* spores may be present after a wash, but frustratingly little information

was provided as to the number of spores and references for following up this information.

1.2.1.1 Infected linen wash cycle conditions

The process and conditions for fouled or infected linen, state that the main wash cycle must reach a minimum of 71°C for ≥ 3 minutes, or 65°C for ≥ 10 minutes with additional mixing time based on fabric to water ratio (Department of Health, 2016a), thus, an additional 4 minutes for $<0.056\text{kg/litre}$ and 8 minutes for $>0.056\text{kg/litre}$ are required. In addition, infected linen must be submitted to a separate pre-wash, so as not to pose a health risk to workers should a blockage occur in the pre-wash section of a Continuous Tunnel Washer (CTW). The choice of detergent is not governed by the policy, neither is the length or number of rinses or the rinse water temperature, leading to significant variation between laundry sites, which still meet the minimum quality requirements.

1.2.2 Survival of indicator organisms in healthcare laundries

There are studies which can provide some support for the advised time and temperature conditions relating to the reduction of bacterial or fungal load on used linen. For example, Orr et al. (2002) demonstrated the thermotolerance of *Enterococcus faecium* and *Enterococcus faecalis in vitro* at commonly used healthcare temperatures, both organisms are considered to be relatively

thermotolerant. Of the 40 strains used, 15 strains exhibited $<3 \log_{(10)}$ reduction after 3 minutes at 71°C and 4 strains had $<3 \log_{(10)}$ reduction after 10 minutes at 65°C. Interestingly a $>5 \log_{(10)}$ reduction was seen in only two strains after 3 minutes at 71°C and two other strains after 10 minutes at 65°C. However, when the strains were inoculated onto strips of cotton and washed in one hospital laundry, with infected linen, $\geq 5 \log_{(10)}$ reduction was seen in all strains. Twelve strains were taken forward and tested in ten hospital laundries, to account for different methods employed in applying HSG (95) 18. For all 12 strains, an average of $\geq 6.6 \log_{(10)}$ reduction was demonstrated across all laundries after just the washing cycle, with original time-zero inoculation of between 7-8.5 $\log_{(10)}$ cfu/sample. Furthermore, in 6/10 laundries enterococci could not be isolated after a full cycle, even when the samples were processed by enrichment culture. Of the other four laundries, one was negative for enterococci after washing only and after a combined wash/dry cycle, the enrichment was negative. Two laundries had positive enrichment cultures after washing and only one laundry had two strains survive the full cycle including calendaring (heated rollers used to flatten and finish linen).

The results suggest that the combination of the thermal disinfection and dilution/mechanical removal in an infected linen wash were able to successfully decontaminate strips of cotton artificially contaminated with enterococci. A major limitation was the lack of typing of the isolates found after washing. It

would have been informative to compare the isolates with the original inoculated strain. Several previously sterile strips, included with the inoculated strips, were found to be positive, which suggests cross-contamination either between strips or from other linen in the wash cycle.

Fijan *et al.* (2007) assessed laundry conditions in an Electrolux washcator using indicator organisms *Enterococcus faecium*, *Staphylococcus aureus*, *Mycobacterium terrae*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa* and *Candida albicans*. Artificial soiling was used to replicate human faeces; the authors chose artificial sweat, swine blood and swine fat. The bioindicators were inoculated onto cotton swatches and replicates were sampled after each stage. There was no significant difference in the antimicrobial effects of washing, between each of the artificial soiling substrates. All indicator organisms survived both a 35°C and 45°C pre-wash type cycle with detergent alone, demonstrating low wash temperatures are not adequate even for relatively temperature-sensitive vegetative cells.

E. faecium, *S. aureus*, *E. aerogenes* and *P. aeruginosa* were able to survive after a main wash cycle where the temperature reached but was not held at 60°C. However, only *E. faecium* survived the cycle which included a pre-wash at 35°C, followed by fresh water and a main wash which reached, but was not held at 60°C. These findings suggest that removal of bacterial cells, thermal

disinfection, dilution and flushing between cycles was a crucial part of the decontaminating effect of that particular cycle.

The 75°C wash cycle, which included a pre-wash at 35°C and a main wash which was held at 75°C for 9 minutes, was successful at reducing bacterial cell and fungal load by 100%. The pre-wash had detergent 6.2g/Kg textiles and the main wash had 5g/Kg textiles detergent and 4.4 ml/Kg textiles bleaching agent. There was repeated addition of detergent and flushing of the water which may have aided removal of the cells.

Interestingly, the disinfection cycle alone was also successful at reducing bacterial and fungal load by 100%. The disinfection cycle was a 2 minute cycle at 80°C with a disinfecting agent at 3ml/Kg textiles, without a pre-wash or main wash. Soiling was not shown to have a protective effect, when thermal and chemical disinfection were used together in a full cycle. Clearly, the combination of thermal and chemical disinfection, alongside the mechanical removal was important in the successful decontamination of the organisms studied.

The authors conclude that *E. faecium* was the most heat-resistant species used in this study and work is needed on viruses and spore forming species such as *Bacillus* spp. due to the thermotolerance of spores from this genus. It also stands to reason that other spore-forming species, which may pose a health risk in a healthcare environment should also be considered. *C. difficile* spores exhibit

in vitro resistance to temperatures much higher and for much longer than the healthcare laundry policy requires e.g. 90°C for up to 10 minutes (Rodriguez-Palacios *et al.*, 2010; Rodriguez-Palacios and LeJeune, 2011).

1.2.3 Survival of *C. difficile* spores in healthcare laundries

Several small studies have looked at the efficacy of the healthcare industrial wash parameters, to inactivate or remove *C. difficile* spores. Hellickson & Owens (2007) found cross-contamination occurred during laboratory-based simulated washes, using sterile swatches and swatches inoculated with $\sim 4 \log_{(10)}$ *C. difficile* (ATCC 9689) spores. The swatches were washed with commonly used detergent and bleach additives (50 ppm chlorine, 54 ppm peracid or 100 ppm peroxide). Both sterile and inoculated swatches were sampled post-wash and were contaminated with viable spores. *C. difficile* spores recovered from fabrics were recorded as presence or absence of growth in media, with no quantification. Despite the lack of detail regarding the agents used and the quantity of spores recovered, these preliminary results suggest a capacity for *C. difficile* spores to survive the temperatures, low-level hypochlorite additives and dilution processes of the infected linen wash. There was no mention of simulated soiling, which could influence the effectiveness of the detergents used and potentially have a protective effect (Diab-Elschahawi *et al.*, 2010).

Lakdawalla *et al.* (2011) found that *C. difficile* spores were recovered from labelled linens washed in a CTW at 71°C for ≥ 3 minutes. A total of $10^1 - 10^3$ cfu/100cm² was found on linen washed as non-infected. The ribotypes of the isolates from linen after washing, matched the isolates from the CDI patients who had provided the linen, except one sheet which had an additional ribotype. The fact that the positive samples were from sheets washed as standard linen, is of concern. It is not clear why the infected sheets were not put through a separate pre-wash. This study did show the survival of spores on sheets which have been washed in the main CTW cycle. The original contamination loads were unknown, which could be an important factor. For example, if the original spore loads were low e.g. 3 - 4 log₍₁₀₎ cfu/100cm² the reduction was minor and could indicate that there might be a greater level of survival if there were higher original loads in other batches. Should the original spore loads have been 7-8 log₍₁₀₎ spores/100cm², the wash process could have been deemed to be very effective. In addition, other sheets with a lower spore load could conceivably be entirely decontaminated.

Another key piece of missing information is whether the samples were taken from sections which were known to have been soiled, or from a selected region i.e. the central region where a patient may have been positioned. It is likely that the number of spores present depends on where on the sheet the sample was taken. Although cross-contamination was shown, the position of

soiling and where samples were taken from are important to get an accurate measure of spore load (Hellickson and Owens, 2007).

Towels and linen may also harbour *Bacillus cereus* spores, with evidence that they survive the laundry process when laundered according to the guideline available. In work carried out by Barrie *et al.* (1994) linen was processed in a CTW, which again demonstrated that spores were able to survive, albeit this study was investigating the genus *Bacillus* (Barrie *et al.*, 1994).

There is some evidence that *C. difficile* spores may survive the minimum wash conditions determined by the department of health, so it is necessary to understand the intrinsic characteristics of the spores which may contribute to their survival in the wash.

1.3 *C. difficile* spore tolerance of industrial wash conditions: agitation, temperature and detergent

C. difficile is an obligate anaerobic organism, the vegetative cells require a strict oxygen-free atmosphere for outgrowth in the laboratory setting. Jump *et al.* (2007) demonstrated survival of *C. difficile* vegetative cells in room air for 15 minutes on a dry surface and up to 6 hours on moist surfaces. *C. difficile* is of particular concern, due to the production of endospores, which are resistant to

air, heat, desiccation, UV and most non-bleach based disinfectants (Jump *et al.*, 2007; Maillard, 2011; Ali *et al.*, 2016).

C. difficile spores facilitate survival through otherwise lethal changes to the environment, such as evacuation from the colon, antibiotic usage, environmental disinfectants and cleaning (Wilcox, 2003). Spore characteristics including the innate resistance of *C. difficile* spores to decontamination have been under-investigated despite the contribution of *C. difficile* to HAI rates (Maillard, 2011; Paredes-Sabja *et al.*, 2014; Fraise, 2015). This is likely to be because of the difficulties associated with obtaining a spore suspension with sufficient *C. difficile* spore titre and the limited relevance of disinfectant exposure tests to a practicable hospital cleaning protocol (Maillard, 2011; Fraise, 2011; Fraise, 2015). Spore-related research has instead tended to focus on *Bacillus subtilis* which has been well characterised, with focus shifting onto *Bacillus anthracis* and *Bacillus cereus* in recent times due to bioterrorism and food security agendas (Setlow, 2007; Paredes-Sabja *et al.*, 2014). In addition, *Clostridium perfringens* and *Clostridium sporogenes* have been studied to give a better understanding of the variation between the spore-forming species. Where relevant and necessary, studies focused on other species of spore-forming bacteria were considered, due to a lack of published work regarding the specific nature of *C. difficile* spores.

1.3.1 Spore structure

The innate resistance of endospores to decontamination processes, such as chemicals or heat, is due in large part to their physical structure, low water content and small acid-soluble proteins (SASPs) which protect spore DNA (Russell, 1998; Paredes-Sabja *et al.*, 2014; Gil *et al.*, 2017). The DNA, RNA and enzymes are present within a dehydrated core, contained within the cortex which in turn, is covered by the spore coat (Setlow, 2007). Figure 1.1 provides a simplified diagram of the main components of a typical spore.

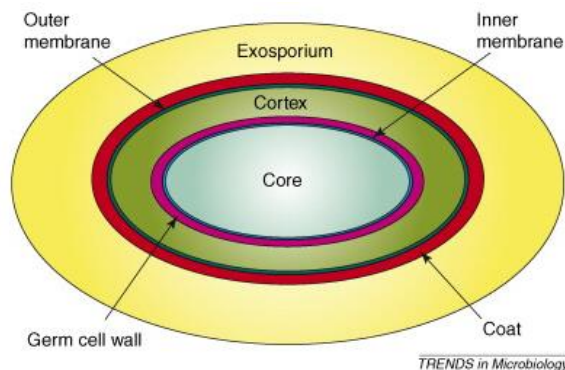


Figure 1.1. The structure of bacterial endospores (Setlow, 2007), reproduced with permission from Elsevier.

The spore coat restricts the entrance of large molecules into the spore, particularly toxic substances and in some cases, such as *C. difficile*, it is overlaid by an outer membrane called the exosporium. Although the overall spore structure is similar in *C. difficile* when compared with other *Clostridium* and

Bacillus spp., there are significant differences in the composition, sporulation and germination of *C. difficile* spores compared to other spore-forming organisms (Paredes-Sabja *et al.*, 2014; Gil *et al.*, 2017). Proteomic study of the *C. difficile* spore has identified 300 spore-associated proteins, half of which have no known homolog in other spore-forming species (Lawley *et al.*, 2009; Abhyankar *et al.*, 2013; Díaz-González *et al.*, 2015). Work continues to determine the purpose and structure of the specific *C. difficile* spore proteins, particularly exosporium proteins for use as vaccine targets (Ghose *et al.*, 2016).

1.3.2 Spore thermotolerance

It has been established that endospore resistance to heat is related to the low water content of the spore core, although the protective mechanism is not clear (Paredes-Sabja *et al.*, 2014). The water content in a vegetative cell can range from 75-80%, whereas in the spore it may be 27-55% depending on bacterial species. In addition, the presence of Dipicolinic acid (DPA) within the core also has a bearing on heat resistance (Paredes-Sabja *et al.*, 2014). DPA is present in the spore cores of both *Bacillus* and *Clostridium* spp. comprising 5-15% of the spore weight. SASPs also play a role in resistance to UV damage to spore DNA, they make up 6% of total spore protein and are highly conserved between *Bacillus* and *Clostridium* spp.

The temperature at which *C. difficile* spores are inactivated is an important consideration when attempting to decontaminate healthcare textiles. There are few published studies which specifically assess the moist-heat resistance of *C. difficile* spores. Although with the potential for foodborne transmission of *C. difficile*, several studies have focused on the presence of spores in meat and the effectiveness of cooking temperature guidelines for inactivating *C. difficile* spores (Rodriguez-Palacios *et al.*, 2010; Hoover and Rodriguez-Palacios, 2013).

Rodriguez-Palacios *et al.* (2010) quantified the inhibitory effect of heating *C. difficile* spores to 71°C, a common food-heating guide temperature, and also a key temperature proscribed by the NHS laundry policy for decontamination of linen (Department of Health, 2016a). The 20 strains investigated were mainly food-derived strains collected from bovine or meat sources, due to the authors being concerned with meat products as a source of CDI. These included seven ribotypes, four of which were of relevance in human disease and international epidemics; ribotypes 001, 014, 017 and 027. The spores of all 20 strains of *C. difficile* survived for 2 hours at 71°C. Subsequently, to look at the effect of re-heating, non-heated spores were heated to 71°C for 30 minutes, then re-heated to 85°C. Interestingly, 90% of strains were unable to survive for 10 minutes after reheating at 85°C and all spores were eliminated after reheating for 20 minutes at 85°C.

To follow up, Rodriguez-Palacios *et al.* (2011) quantified the effects of moist-heat effect at temperatures up to 96°C. Results showed that moist heat at 85°C inhibited spore recovery by up to 6 log₍₁₀₎ within 15 minutes in most, but not all the strains tested. Ultimately, when samples containing less than 4 log₍₁₀₎ spores were heated to 85°C there were no cultivable spores after 15 minutes. Heating aged spores at 63°C and 71°C showed increased recovery of 30% over non-heated samples, which the authors suggest might be due to reactivation of 'superdormant' spores.

Alfa *et al.* (2008) looked at thermal disinfection of *C. difficile* spores using bed pan washers. The decontamination cycle on the experimental ward Washer-Dryer (WD), reached 80°C for 1 minute, this proved to be inadequate for inactivating *C. difficile* spores in faeces, held within a cryovial. The decontamination temperature was increased to 85°C for 5 minutes and the rinses were changed from 2 cold/3 warm rinses to 4 cold/4 warm water rinses, which resulted in undetectable levels of *C. difficile*.

A similar method using bedpan washers was assessed by Macdonald *et al.* (2016) including 9 cryovials of spores and 45 artificially contaminated bed pans. The bedpans were inoculated with 6 log₍₁₀₎ spores in faecal emulsion, then processed in the decontamination cycle. The cycle consisted of a 5 seconds cold water rinse followed by 5 seconds warm water rinse, 5 minutes warm water

wash with detergent, 15 seconds hot water rinse, ending with thermal disinfection via steam at 91°C for 1 minute. The detergent was an alkali product with pH12. After a full calibrated cycle the bedpans had either one cfu (1/45), two cfu (1/45) or no viable spores (43/45). The cryovials showed reductions in viable spores ranging from 1-4 log₍₁₀₎ cfu/ml reduction from the original 7 log₍₁₀₎. This study highlights the inability of temperature alone to inactivate *C. difficile* spores and of the need for detergent and multiple rinses to remove spores from inanimate surfaces. However, due to bed pans having smooth hard surfaces, there may be a greater chance of removal of spore contamination during their respective wash cycles compared to linen.

1.3.3 Spore resistance to detergents and disinfectants

The sporicidal activity of disinfectants has been tested using various standards, for example BSEN 13704, BSEN 14347 and a recent test standard proposed by Fraise *et al.* (2015), which is accredited by Public Health England (British Standards Institution, 2002; British Standards Institution, 2005; Fraise *et al.*, 2015; Wesgate *et al.*, 2016). Due to the variation in test standard followed, method of spore culture and spore purification there is a lack of consistency and no single standardised test.

To address the variation in test protocols Wesgate *et al.* (2016) compared four sporicidal activity test standards, using *B. subtilis* and *C. difficile* (11209)

spores. Instead of preparing the spores as recommended in each test, spore preparation was standardized and then each test standard was followed rigorously for contact times of 5 and 60 minutes. The author tested eight chemical compounds including glutaraldehyde, two oxidizing agents and due to a well-established sporicidal activity, sodium hypochlorite (5000 ppm) was used as a positive control. A reduction of $>4 \log_{(10)}$ spores was considered a successful demonstration of sporicidal activity. After 5 minutes exposure sodium hypochlorite was sporicidal against *C. difficile* spores using all 4 test methods. However, against *B. subtilis* spores, the sodium hypochlorite was only sporicidal at 60 minutes. The two species showed a statistically significant difference in spore susceptibility to sodium hypochlorite. Glutaraldehyde was not sporicidal at either test time; OPA was not sporicidal against *B. subtilis* at either test time, but was sporicidal against *C. difficile* after 60 minutes. The two oxidizing compounds were sporicidal at 60 minutes against the spores of both species. The overall $\log_{(10)}$ reduction varied depending on the test method used and sometimes the compounds failed at 5 minutes exposure in several tests.

A $>5 \log_{(10)}$ reduction in viable spores has been proposed as an ideal standard for stringent disinfection (McDonnell and Russell, 1999; Fraiese, 2011). However, the British standard BS EN 13704:2002 (BS EN 13704:2002 Chemical disinfectants: Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectants used in food, industrial, domestic and

institutional areas) requires only a $>3 \log_{(10)}$ reduction and requires a contact time of 60 minutes, which is unrealistic in most settings (British Standards Institution, 2002; Wesgate *et al.*, 2016). Only bleach-based products have shown anywhere near this level of sporicidal activity (McDonnell and Russell, 1999). In a laundry wash cycle bleach is used at levels which are not sporicidal and purely to remove stains from the linen. Additionally, soiling in the wash cycle may inactivate bleach-based products and provide a protective effect from heat inactivation (Diab-Elschahawi *et al.*, 2010).

When looking at the sporicidal activity of detergents, there is a single brand which claims compliance with BS EN 13704, Halo laundry detergent, which claims to be effective against *C. difficile* spores at 30°C in a standard domestic wash cycle (A and A Marketing, 2011). To make a sporicidal claim the active ingredient hygienilac, must have been investigated and found to cause a $>3 \log_{(10)}$ reduction in viable *C. difficile* spores after 60 minutes exposure at 30°C.

The effectiveness of industrial laundry products is commonly tested as part of a test of the effectiveness of the whole laundry process including heat, chemicals (such as detergent, bleach and peracetic acid) and rinsing. The overall test of the industrial process is performed by destructive sampling of laundered items on a regular basis in line with the management and provision chapter of the HTM 01-04 (Department of Health, 2016a).

It is of note that one environmental detergent, has been shown to induce sporulation in *C. difficile* vegetative cells, which may add to spore burden in the hospital environment (Wilcox and Fawley, 2000). Wilcox and Fawley (2000) found that a neutral environmental detergent, Hospec, increased sporulation in exposed vegetative cell suspensions *in vitro*.

1.3.4 Spore adherence to substrates

Spores present in the environment need to be able to persist on a substrate to be available to infect a potential host. Adherence of *B. anthracis* spores to soil (earth) has been shown to vary, where adherence of the spores was particularly improved in soil containing large proportions of organic matter and when the exosporium layer was removed from spores (Williams *et al.*, 2013).

The well characterised spores of *B. cereus* and *B. anthracis* have a loose fitting exosporium layer with hair-like projections (Henriques and Moran, 2007; Paredes-Sabja *et al.*, 2014; Pizarro-Guajardo *et al.*, 2016). In contrast, most *C. difficile* strains produce spores with an exosporium layer that is tightly attached to the spore coat, while also having hair-like projections; the spores of *C. difficile* strain 630, have a hair-less electron-dense exosporium-like layer that is also attached to the spore coat (Barra-Carrasco *et al.*, 2013; Pizarro-Guajardo *et al.*, 2016). The presence of *C. difficile* specific proteins in the exosporium layer, such as *C. difficile* exosporium cysteine-rich protein (CdeC), suggest there are

significant differences in the structure and perhaps function of *C. difficile* spore layers in contrast to spores from *Bacillus* spp. (Barra-Carrasco *et al.*, 2013). The exosporium layer in both *Bacillus* spp. and *C. difficile* spores contributes to hydrophobicity and both types of exosporium exhibit collagen-like exosporium glycoproteins (Brahmbhatt *et al.*, 2007; Joshi *et al.*, 2012; Escobar-Cortés *et al.*, 2013; Pizarro-Guajardo *et al.*, 2014).

Panessa-Warren *et al.* (1997) used SEM to observe the exosporium of *C. difficile* spores. The usually smooth exosporium layer of a dormant spore, developed a 'bumpy' texture consisting of small projections once activated. The appearance of the projections was associated with adherence to an agar substrate and resistance to removal by agitation. A single thick appendage developed from the exosporium, which aided further attachment to the substrate and co-agglutination with nearby spores. At maximal adherence, after 105 minutes, spores were able to resist removal during malachite-green staining, rinsing in running water and removal by agitation in water, or colchicine, at 300rpm. Interestingly, *C. difficile* ATCC 43594 spores exhibited reduced adherence when static, but increased adherence when agitated in water, which the authors suggest may be adaptive of the conditions in the hypermotile colon (Panessa-Warren *et al.*, 1997).

1.4 In summary

The evidence from genetic relatedness of CDI strains suggests there are unknown sources of non-hypervirulent *C. difficile* spores, which are causing up to 82% of CDIs in vulnerable patients. The intrinsic characteristics of *C. difficile* spores, mean they are particularly difficult to inactivate using detergents and the presence of soiling can inactivate bleach-based products which are currently used in environmental cleaning. The industrial laundry cycle parameters, including time and temperature, are such that in principle *C. difficile* spores could tolerate the decontamination process. Evidence of survival and cross-contamination of *C. difficile* spores in simulations and preliminary studies also suggest that these industrial wash parameters are inadequate. The failed decontamination of laundry could be contributing to surface contamination within hospitals, particularly in non-CDI specific hospital wards which do not employ specific control and isolation measures. Due to the rented linen business model, sheets processed at a regional commercial laundry facility can be distributed to other hospitals within the region. There is a real need to investigate the laundry parameters and their ability to provide safe and microbiologically clean linen.

1.5 Scope of the work

1.5.1 Overall Aim

The overall aim of this research was to quantify, and investigate the factors influencing, the survival of *C. difficile* spores on healthcare linens washed in the conditions specified by the NHS laundry policy, HTM 01-04 Decontamination of linen for health and social care.

1.5.2 Principal objectives

1. To determine suitable media and methods for growth, sporulation and germination of *C. difficile* cells and spores (Chapter 2).
2. To assess the efficacy of the HTM 01-04 wash cycle for decontamination of NHS (100% cotton) sheets contaminated with *C. difficile* spores (Chapter 3).
3. To evaluate time, temperature and detergent parameters, to effectively decontaminate linen contaminated with *C. difficile* spores (Chapter 4).
4. To determine the role of the exosporium in adherence of *C. difficile* spores to NHS (100% cotton) sheets (Chapter 5).

2 Culturing and sporulation techniques for *C. difficile*

2.1 Chapter abstract

C. difficile is a Gram-positive, anaerobic bacterial species. The hardy spores are produced within hours of infection *in vivo*, however sporulation is more difficult to achieve *in vitro* at levels suitable for investigations. The aim of this study was to ascertain a suitable sporulation method, for the purpose of generating spore suspensions of between 5 and 8 log₍₁₀₎ cfu/ml. Sporulation was induced and quantified in cooked meat broth (CMB) cell cultures using a variety of stressors (aerobic incubation, exposure to alcohol and long incubation). The viability of spore suspensions was assessed after 30 days cold storage at 4°C. The sporulation method utilising aerobic stress of CMB cultures, was superior to alcohol stress of CMB when producing spore suspensions, yielding 7.77 log₍₁₀₎ cfu/ml and 5.36 log₍₁₀₎ cfu/ml, respectively ($p \leq 0.05$). An additional long incubation (14 days in CMB) method was developed for subsequent investigations, when pooled there was an average 8.06 log₍₁₀₎ cfu/ml; similar to other published batch culture methods. The method of generating spore suspensions chosen for further investigations, was the long incubation (14 days in CMB) method. Storage did not affect the viability of the spore suspensions, with no difference when enumerated immediately and after 30 days cold storage ($p > 0.05$). Storage for 30 days had no effect on spore suspension viability.

2.2 Introduction

C. difficile is primarily a mammalian gut pathogen and is frequently cultured from livestock, companion animals and humans, often when presenting with diarrhoea (Songer and Anderson, 2006; Rupnik, 2007; Goorhuis *et al.*, 2007; Schoster *et al.*, 2012; Bailey *et al.*, 2016). *C. difficile* is an obligate anaerobic organism and vegetative cells require a strict oxygen-free atmosphere for growth (Sorg and Dineen. 2009; Edwards *et al.*, 2013). Initially, Buggy *et al.* (1983) found that vegetative *C. difficile* cells were inactivated after 15 minutes in room air on a dry glass slide.

This was further investigated by Jump *et al.* (2007) where vegetative cells were shown to survive 15 minutes in room air on a dry glass slide and on a dry slide incubated anaerobically at 37°C. The rapid inactivation in optimal anaerobic and temperature conditions suggested it was the effect of drying that inactivated the cells, as opposed to air exposure as suggested by Buggy *et al.* (1983). Subsequently, vegetative cells have been shown to survive in moist conditions for much longer, up to 6 hours in room air on a moist glass slide and up to several weeks in refrigerated buffered samples at 4°C (Freeman and Wilcox, 2003; Jump *et al.*, 2007).

In vitro, anaerobic growth conditions for culturing *C. difficile* cells are best achieved using an anaerobic cabinet, with an anaerobic gas mixture (10% Hydrogen, 10% Carbon Dioxide and 80% Nitrogen), a catalyst such as palladium

to remove oxygen and a compound which reduces volatile fatty acids produced during anaerobic metabolism (Edwards *et al.*, 2013). Vegetative *C. difficile* cells require a stable 37°C, at which they will grow very rapidly, producing visible colonies within 18 hours of inoculation on to media (Sorg and Dineen, 2009).

The optimal pH of the media is between pH 7.8 - 8, mild acidic and alkaline conditions are well tolerated, but moderate acid conditions inhibit *C. difficile* vegetative cell growth (Sorg and Dineen. 2009; Perez and Springthorpe, 2011; Scaria *et al.*, 2014). *C. difficile* cells require six essential amino acids for fermentation, metabolism and growth—leucine, isoleucine, proline, tryptophan, valine, and glycine, but different media can affect the order and amount of each amino acid used (Neumann-Schaal *et al.*, 2015). In amino-acid rich media, the favoured sources of carbon and energy are proline, leucine and cysteine, whereas glutamate and lysine are rarely or not used. Unsurprisingly, *C. difficile* does not utilise simple sugars as a carbon source, as they are not found in the large intestine (Ferraris *et al.*, 1990; Neumann-Schaal *et al.*, 2015).

The vegetative cells form a hardy endospore as a survival strategy and enables transmission of CDI (Borriello, 1998; Giel *et al.*, 2010). *C. difficile* cell cultures are induced to sporulate in liquid media or on solid media to enable the recovery of spore rich suspensions for further investigation (Hasan *et al.*, 2011; Alfa *et al.*, 2013; MacDonald *et al.*, 2016).

The *C. difficile* vegetative cells produce spores in response to external stimuli, perhaps due to stresses such as reduced nutrients, aerobic conditions or increasing levels of metabolic waste products; although in a mouse model sporulation can occur within 6 hours of infection (Sorg and Dineen, 2009; Koenigsknecht *et al.*, 2015). Furthermore, it has been demonstrated that spores are formed within 12 hours of initiation of sporulation (Pereira *et al.*, 2013).

The spores may be harvested by various means such as the use of cell scrapers removing visible growth on agar or centrifugation of broth cultures (Hasan *et al.*, 2011). During protocols designed to induce sporulation for spore harvesting, vegetative *C. difficile* cells are commonly inactivated by heating liquid cultures to 80°C for 10 minutes or by exposing to alcohol for 20 mins (Merrigan *et al.*, 2010; Rodriguez-Palacios *et al.*, 2010; Rodriguez-Palacios and LeJeune, 2011; Alfa *et al.*, 2013; MacDonald *et al.*, 2016). The thermotolerance of *C. difficile* spores, investigated in regard to cooking guidelines, has been shown to exceed 2 hours at 71°C (Rodriguez-Palacios *et al.*, 2010). *C. difficile* spores have been shown to survive in cryovials exposed to 91°C- 93°C for 1 minute, where after wash survival was variable between 2-4 log₍₁₀₎ cfu/ml viable spores per cryovial; demonstrating that a high temperature alone may not be enough to consistently inactivate spores (MacDonald *et al.*, 2016).

C. difficile spores exhibit no susceptibility to UV, desiccation or oxygen and can survive on surfaces in room air for many months, but possibly for years (Kim *et al.*, 1981; Wilcox *et al.*, 2003; Ali *et al.*, 2016; Gil *et al.*, 2017). Spores have additional germination requirements to resume metabolic activity, for example the main germinant is cholate, but a derivative, taurocholate, is more commonly used in germination media (Sorg and Sonenshein, 2008).

Once a *C. difficile* spore suspension has been produced, it needs to be kept in cold storage to maintain viability of the spores. Cold storage of *C. difficile* spore suspensions has demonstrated variable results on the viability of spore suspensions over time (Freeman and Wilcox, 2003; Rodriguez-Palacios and LeJeune, 2011).

2.2.1 *C. difficile* cell culture: available methods

C. difficile was named as such due to the difficulty in culturing perceived by the scientific community at the time of discovery (Hall and O'Toole, 1935). It is apparent from the published literature that there are few standardised methods which can be applied to study *C. difficile* cell cultures and to generate spore suspensions (Bartlett, 2009; Hasan *et al.*, 2011; Edwards *et al.*, 2013; Fraise *et al.*, 2015).

At present there are many different media available to aid culturing *C. difficile*. Solid media, includes commercially manufactured agar plates such as

Centres for Disease Control (CDC) anaerobic 5% sheep blood agar (CABA), Egg yolk agar, colombia agar (CA) and cycloserine-cefotaxime fructose agar (CCFA) (Hasan *et al.*, 2011). These agars are prohibitively expensive to use on a large scale project. As an alternative a base agar can be supplemented to make them suitable for *C. difficile* growth (Sorg and Dineen, 2009). Common base agars include brain heart infusion (BHI) agar, Brazier's agar, *Clostridium difficile* agar base or fastidious anaerobe base (Bartlett, 1994; Sorg and Sonenshein, 2008; Bartlett, 2009; MacDonald *et al.*, 2016), which are then supplemented with various compounds, such as yeast extract, cysteine or blood to make them suitable for culturing *C. difficile* (Bartlett, 2009; Sorg and Dineen, 2009).

The main types of blood used are sheep or horse blood (Marsh *et al.*, 2006; Didelot *et al.*, 2012; MacDonald *et al.*, 2016). The media (solid agar plates and broth) need to be reduced before use to remove dissolved oxygen. This is achieved by maintaining the media in an anaerobic environment with a catalyst, usually palladium, for 2-24 hours prior to inoculation (Citron, 1984; Edwards *et al.*, 2013). Growth on solid and in liquid culture has been achieved with a variety of media (Table 2.1).

Table 2.1. Various growth media for culturing *C. difficile* cells.

Source	Solid Agar	Broth media
Marsh <i>et al.</i> (2006)	Sheep blood agar	Tryptone soy broth
Bartlett (2009)	BHIS*(with cysteine and yeast)	BHIS*(with cysteine and yeast)
Sorg (2009)	BHIS*(with cysteine)	BHIS*(with cysteine)
Hasan <i>et al.</i> (2011)	CABA**	Columbia and BHI ⁺
Didelot <i>et al.</i> (2012)	Columbia Blood Agar	Nutrient broth
Ali <i>et al.</i> (2016)	Columbia Blood Agar	N/A

*Brain heart infusion (with supplements)

**Centre for Disease Control anaerobic sheep blood agar

+Brain heart infusion

To understand the growth dynamics of different strains, Vohra and Poxton (2011) assessed multiple strains using a spectrophotometer to measure culture turbidity at OD₆₀₀ over 12 hours. The authors were able to give an estimate of turbidity, but little idea of how the turbidity related to the number of viable cells and spores. Therefore, due to problems with quantification by light absorbance, spread plating and direct enumeration are the preferred method.

2.2.2 Sporulation in *C. difficile* cell cultures

The specific signal to initiate sporulation in *C. difficile* vegetative cells is unknown at present, but is likely due to environmental stress, nutrient

starvation and an adaptive strategy for persistence in a host (Higgins and Dworkin, 2012; Paredes-Sabja *et al.*, 2014). In *B. subtilis* cultures, it has been shown that when sporulation is initiated, the vegetative cell develops a polar septum during asymmetric cell division, resulting in a small forespore and a large mother cell (Higgins and Dworkin, 2012; Mckenney *et al.*, 2013). The forespore is then engulfed by the mother cell, followed by metabolic dormancy, DNA compaction and finally the formation of the outer layers: cortex, coat and exosporium (Henriques and Moran, 2007; Mckenney *et al.*, 2013).

The exact period of time required for complete sporulation in a *C. difficile* vegetative cell is not clear; the latest estimates suggest 12 hours from initiation to sporulation, however sporulation can be heterogeneous and asynchronous (Pereira *et al.*, 2013). Burns *et al.* (2010) demonstrated complete sporulation in a culture after 120 hours, with maximal spore concentration of 14 *C. difficile* strains of between 5-7 log₍₁₀₎ cfu/ml. The sporulation rates of different strains exhibited statistically significant variation, which was not dependent on type e.g. hypervirulent vs non-hypervirulent types (Burns *et al.*, 2010). A *C. difficile* sporulation media (SM) was first described in Wilson *et al.* (1982) and used in Pereira *et al.* (2013) to assess *C. difficile* cell culture sporulation over 24 hours – 72 hours, the maximal sporulation achieved in 72 hours was an average of 4.7x10⁶ cfu/ml (6.67 log₍₁₀₎ cfu/ml). Vegetative *C. difficile* cells were inactivated by heat treatment. The authors compared sporulation media (SM) to BHI broth

and found SM to result in a higher spore concentration, although the data was not shown.

Merrigan *et al.* (2010) took samples of BHI broth cultures, at 8, 20, 24 and 48 hours anaerobic incubation. Samples were heat treated to inactivate vegetative cells and enumerated on Taurocholate fructose agar (TFA) resulting in spore concentrations of $1-1.2 \times 10^7$ cfu/ml ($7-7.1 \log_{(10)}$ cfu/ml). It has also been shown that in *B. subtilis*, that the growth media can affect sporulation rate, either inhibiting or increasing sporulation depending on composition and availability of nutrients (Errington, 1993). Without any additional manipulation, a broth culture of *C. difficile* cells can be expected to have an increase in the concentration of spores over the first 120 hours to between 5 and 7 $\log_{(10)}$ cfu/ml (Burns *et al.*, 2010; Merrigan *et al.*, 2010; Perez and Springthorpe, 2011). However, the final spore concentration may depend on the *C. difficile* strain and type of media used.

2.2.2.1 Sporulation methods

There are two main approaches to inducing or increasing sporulation in *C. difficile* cultures, by the stressing of cells growing on either solid agar or in liquid broth cultures. The sporulation methods utilise a stressor such as long-incubation, an aerobic environment or alcohol to induce sporulation in *C. difficile* vegetative cells.

Wullt *et al.* (2003) used a 48-hour plate culture, which was transferred to BHI broth for 48 hours and then alcohol treated for 30 minutes at room temperature. The spores were recovered by unknown means and *C. difficile* spore concentration in the resulting suspensions were 3×10^5 - 2×10^6 cfu/ml (5.48 - $6.30 \log_{(10)}$ cfu/ml). The comparatively low concentration of spores obtained could be attributed to the short culture time, short contact time with the alcohol and rapid harvesting. Alternatively, a low concentration of *C. difficile* vegetative cells would have given fewer cells to undergo sporulation. Gram staining was used to verify that 85-90% of the bacterial structures were spores.

Hasan *et al.* (2011) evaluated several solid and liquid media types for optimum sporulation. The authors concluded that using pooled suspensions scraped from seven CABA plates incubated anaerobically at 37°C for 7- 10 days gave the highest spore concentration of the methods studied. The CABA plate method generated an average spore concentration of $\sim 8 \log_{(10)}$ cfu/ml.

Other groups have developed their own methods to produce a spore suspension to provide a standardised approach for specific investigations such as assessing sporicidal activity. For example, Perez *et al.* (2011) formulated a new medium, Clospore, which promoted sporulation in *C. difficile* cell cultures. After several rounds of optimisation of the media composition, the maximum spore concentration of 1.6×10^8 cfu/ml ($8.2 \log_{(10)}$ cfu/ml) was recovered from

500ml batch cultures. The Clospore medium was developed for use in a sporicidal agent efficacy test standard, described by Fraise *et al.* (2015).

Alfa *et al.* (2013) inoculated blood agar plates with *C. difficile* cells and incubated anaerobically at 37°C for 14 days, with vegetative cells inactivated by the addition of 95% alcohol to seven pooled samples for 20 minutes. Alcohol was used instead of heating, so that spores would not be pre-heated during the recovery and possibly damaged. The spore suspensions were enumerated on *C. difficile* moxalactam norfloxacin (CDMN) agar plates, but final spore concentration was not reported. The suspensions were used for testing thermal decontamination of bed pan washers, diluted in faeces or urine at a final concentration of 1×10^7 cfu/ml ($7 \log_{(10)}$ cfu/ml), therefore, the final spore concentration must have been $>7 \log_{(10)}$ cfu/ml. The same method involving long incubation of inoculated blood agar plates and alcohol inactivation of cells was also performed in Macdonald *et al.* (2016); the average spore concentration was reported to be 1×10^8 cfu/ml ($8 \log_{(10)}$ cfu/ml).

In the U.S. there is a standardised spore suspension protocol available 'SOP MB-28-00 Production of *Clostridium difficile* Spores for Use in Efficacy Evaluation of Antimicrobial Agents' (USEPA, 2013). The standardised method built on the work of Hasan *et al.* (2011). In short, *C. difficile* lyophilised cultures were enriched in reinforced clostridial medium before being transferred to ten

CABA plates. The plates were incubated anaerobically at $36^{\circ}\text{C} \pm 1^{\circ}\text{C}$ for 7-10 days, with spores recovered as per Hasan *et al.* (2011). The final suspensions were heated to $65^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 10 minutes to inactivate cells, with an average spore concentration of $>8 \log_{(10)} \text{cfu/ml}$.

In summary, there are several sporulation methods using long incubation and alcohol stress to improve sporulation rates in cell cultures. Both solid and liquid media have been used to obtain suspensions with high concentrations of spores. An efficient suspension generation method should achieve a $7-8 \log_{(10)} \text{cfu/ml}$ spore concentration to be useful for further investigation and to be comparable to the methods available.

2.2.3 Germination of spores

Germination of *C. difficile* spores is reliant on the presence of cholate derivatives such as taurocholate and the co-germinants L-glycine and histidine (Sorg and Sonenshein, 2008; Wheeldon *et al.*, 2010; Wheeldon *et al.*, 2011). Wilcox *et al.* (1982) made an early observation that the addition of taurocholate to a selective media, increased the recovery of *C. difficile* from surfaces. The estimated improved recovery was between 1.7 and $5 \log_{(10)} \text{cfu/ml}$, although it was not clear whether this was spores or vegetative cells, as there was no inactivation of cells. Sorg and Sonenshein (2008) showed that taurocholate is in fact a germinant, by incubating spores in supplemented BHI broth with

increasing concentrations of taurocholate, before spread plating onto BHI agar without taurocholate. The concentrations of taurocholate ranged from 0.001% to 10%, and a control with 0%, with spores exposed for 10 minutes. Incubation in 10% taurocholate resulted in the recovery of 60% of spores compared to directly spread plating the same spore suspension on BHIS agar containing 0.1% taurocholate. The authors note that with the highest recovery efficiency seen by direct spread plating onto BHIS/T agar, there could be other important features involved that may relate to either a low-dose continuous exposure to taurocholate or the presence of a solid surface on which to germinate. This work also indicated other primary bile salts such as cholate and glycocholate could act as germinants, albeit with much lower rates of germination. Chenodeoxycholate was unable to initiate germination in this study, but it has previously been shown by Wheeldon *et al.* (2008) to initiate germination. When spores were incubated in taurocholate and individual components of the BHIS, glycine was shown to be necessary as a co-germinant. Both taurocholate and glycine co-germinants needed to be present at the same time for germination to occur. These findings are of relevance both when considering the disease process *in vivo* and to ensure successful culturing of *C. difficile*.

Wheeldon *et al.* (2011) demonstrated that sensitivity to germinants varies with strain type and interestingly between spores from the same suspension. The specific receptor on the surface of *C. difficile* spores for sensing

taurocholate is reported to be CspC, a spore coat bound protease (Francis *et al.*, 2013) However, in some strains germination may not proceed when spores are incubated in buffered taurocholate without glycine; this suggests at least one other receptor is involved which requires glycine for initiation of germination in some strains (Heeg *et al.*, 2012; Francis *et al.*, 2013).

2.2.4 Storage of *C. difficile* spore suspensions

The appropriate storage conditions for stock spore suspensions is vital to maintaining viable spore concentrations for further research. Cold storage at 4°C has been shown to affect the viability of stock spore suspensions. For example, Rodriguez-Palacios and LeJeune (2011) found cold storage at 4°C affected spore concentration of non-heated spore suspensions in PBS; median reduction of cultivable spores was 0.28 log₍₁₀₎ cfu/ml by day 18, which was statistically significant. The authors suggest this could be evidence of the spores entering a superdormant state during cold storage. In addition, repeated sampling of heated-treated (85°C) spores showed no effect after storage at 4°C. In contrast, Freeman & Wilcox (2003) found there was no change in non-heated spore counts of *C. difficile* suspensions stored at 4°C over 56 days where alcohol was used to inactivate cells. Interestingly, there was no difference in recovery when samples were taken on one occasion from different replicate suspensions or when sampling was repeated on a single suspension over the storage time. In contrast, an earlier study by Weese *et al.* (2000) suggested a decline over 72

days, with *C. difficile* in a faecal emulsion stored at 4°C aerobically and no decline seen when stored anaerobically. However, no heat or alcohol was used to differentiate between cells or spores. The results were assessed in terms of frequency of *C. difficile* as 'present' or 'absent' and were not quantified. The media didn't appear to contain taurocholate and supplementation was not reported, which means the result was representing cell presence, accounting for the fewer positive samples when stored aerobically.

2.2.5 In summary

There are a number of possible media and protocols available for growing *C. difficile* cells such as BHI agar plates or broth, CABA plates or Clostridial broth enrichment medium. In addition, methods for inducing sporulation and germination of spores varied between using agar plates and liquid broths with stressors including alcohol, long incubation or exposure to air. Inactivating vegetative cells after sporulation, has been achieved by either moist heat or alcohol exposure. Once successfully recovered, the spore suspensions may lose viability or spores may enter a super-dormant state and the concentration of spores may decline. Repeated enumeration of viable spore concentrations of the recovered suspensions may highlight any issues with storage and viability. The viability of the spore suspensions generated is important to ensure any assessments of sporicidal activity are accurate and the effect is due to the test and not a loss in spore viability due to storage.

2.2.6 Scope of the work

2.2.6.1 Aim

The aim of this investigation was to determine an efficient method of culturing and inducing sporulation in *C. difficile* cell cultures, to provide a consistent concentration of viable spores.

2.2.6.2 Objectives

1. To evaluate the growth, sporulation and germination of *C. difficile* on a variety of media.
2. To determine an appropriate method to induce consistent sporulation in *C. difficile*.
3. To assess the effect of storage at 4°C on the viability of *C. difficile* spore suspensions.

2.3 Methods

2.3.1 Microorganism

The type strain *C. difficile* National Culture Type Collection (NCTC) 11209 was used for non-clinical investigations (NCTC 11209, NCTC, UK). All cultures were stored on Cryocare plastic beads in glycerol, at -20°C, with a duplicate set stored at -80°C (TS70AS, Cryocare, UK). Spore suspensions were stored in Maximum Recovery Diluent (MRD) at 4°C for a maximum of 12 months (CM0733, Oxoid, UK). MRD was used for dilution throughout.

2.3.2 Media assessment

2.3.2.1 Media for cell growth

Brain Heart Infusion agar (BHI) was used to support the growth of *C. difficile* vegetative cells prepared as per the manufacturer's instructions (CM1136B Oxoid, UK). Prior to inoculation, the BHI agar plates were pre-reduced for 24 hours, in a bench-top anaerobic cabinet (Minimac, Don Whitley, UK). The anaerobic gas composition was 80% Nitrogen, 10% Carbon dioxide and 10% Hydrogen. The pre-reduced plates were then inoculated using a single cryobead of *C. difficile* NCTC 11209 and incubated anaerobically at 37°C for 48 hours. Anaerobic conditions were confirmed by the addition of an anaerobic test strip, saturated with resazurin solution (59886, Sigma-Aldrich, UK).

BHI agar was then supplemented with 6% D-fructose (F1027 Sigma, UK), 0.1% L-cysteine (C7352-100G, Sigma, UK), both filter sterilised, and 5% horse blood (HB026, TCS, UK); henceforth called BHI supplemented agar (BHIS). Supplements were added aseptically after the rehydrated agar was sterilised by autoclaving (121°C, 15 minutes, 15 psi). Prior to inoculation, the BHIS agar plates were pre-reduced for 24 hours, then inoculated and incubated as previously stated. The investigation was repeated in triplicate on two separate occasions.

2.2.2.2 Media for spore germination and enumeration

To germinate *C. difficile* spores, the BHIS was supplemented with 0.1% sodium taurocholic acid salt (T4009-5g, Sigma, UK) henceforth BHIS with taurocholate (BHIS/T). Prior to use, the BHIS/T agar plates were pre-reduced for 24 hours.

2.3.3 Identification of *C. difficile*

Identification of colonies for enumeration on non-clinical plates was performed visually for the characteristic grey, ground glass appearance of *C. difficile*, longwave U.V. light fluorescence and by the distinctive manure smell. Regular re-testing of stock cultures was performed prior to experimentation, using U.V. fluorescence and latex agglutination test kit (*C. difficile* M4ICE, Microgen, UK). Clinical isolates were identified visually as above and tested by UV longwave fluorescence, with representative colonies tested by latex agglutination test kit. The investigation was repeated in triplicate on two separate occasions.

2.3.4 Growth curves

To assess the standard growth of *C. difficile* vegetative cells in a liquid culture, broth cultures in two kinds of liquid media were sampled at 0, 2, 4, 6, 8, 10, 12, 24 and 48 hours for vegetative cell concentration and at 0, 6, 12, 24 and 48 hours for spore concentration.

2.3.4.1 Cryobead directly into BHI broth

Triplicate 10ml aliquots of pre-reduced BHI broth were inoculated with a single *C. difficile* storage cryobead and incubated anaerobically at 37°C for 24 hours.

The investigation was repeated on two separate occasions.

2.3.4.2 Colony from BHIS culture into BHI broth

C. difficile cryobeads were streaked on two pre-reduced BHIS agar plates and incubated anaerobically at 37°C for 24 hours. Sixteen 10ml aliquots of pre-reduced BHI broth were then inoculated with a single colony of *C. difficile* from the BHIS agar plates. The broth subcultures were incubated anaerobically at 37°C for 48 hours. The investigation was repeated in duplicate on two separate occasions.

2.3.4.3 Colony from BHIS culture into Cooked Meat Broth

C. difficile cryobeads were streaked on two pre-reduced BHIS agar plates and incubated anaerobically at 37°C for 24 hours. Sixteen 10ml aliquots of pre-reduced cooked meat broth (CMB) were each inoculated with a single colony of *C. difficile* from the BHIS agar plates (60865 Sigma, UK). The broths were incubated anaerobically at 37°C for 48 hours and sampled at each time point. The cultures were homogenised by inverting cultures before sampling. Vegetative cell concentration was enumerated by serial dilution in MRD, with spread plating onto pre-reduced BHIS and 48 hour anaerobic incubation. Spore samples were taken by dispensing 1ml aliquots into centrifuge tubes. Vegetative

cells were inactivated by heating to 80°C for 10 minutes in a water bath at 100rpm. The spore concentration was enumerated by serial dilution in MRD spread plating onto BHIS/T and anaerobic incubation at 37°C for 48 hours. The investigation was repeated in duplicate on two separate occasions.

2.3.4.4 Contamination assessment

Heat-fixed smears of 48 hour *C. difficile* broth cultures were stained using differential Schaeffer-Fulton spore stain or Gram stain. Cultures were also streaked onto pre-reduced BHIS agar and incubated either anaerobically for 48 hours or aerobically for 48 hours. Resulting colonies were identified as previously described (section 2.2.3). To distinguish between *Bacillus* spp. and *Clostridium* spp., catalase activity was also assessed using hydrogen peroxide. Definitive identification was subsequently determined using the latex agglutination test kit as per the manufacturer's instructions (section 2.2.3). The investigation was repeated in triplicate on two separate occasions.

2.3.5 Sporulation

Vegetative cell cultures of *C. difficile* were either grown on BHIS agar plates or in liquid CMB culture.

2.3.5.1 Agar-based methods

To prepare the agar plates for the sporulation investigation, duplicate pre-reduced BHIS agar plates were inoculated by streaking a *C. difficile* cryobead

onto the surface and incubating anaerobically at 37°C for 48 hours. *C. difficile* cells were then inoculated onto seven pre-reduced BHIS agar plates to give a lawn of growth. After anaerobic incubation at 37°C for 48 hours. The *C. difficile* cells were exposed to the following stressors to induce sporulation.

Either:

- a. Aerobic stressor – the seven agar plates were incubated aerobically for seven days at 25°C.
- b. Alcohol stressor – the seven plates had 5ml of 70% ethanol added to each culture plate. The alcohol treated plates were left for a further 30 minutes or 3 hours anaerobic incubation.
- c. Temperature stressor – the temperature in the anaerobic cabinet was increased to 40°C for 24 hours.
- d. Long incubation stressor – The seven plates were incubated anaerobically for five days at 37°C.

Spores were then recovered into suspensions by the addition of 2ml of sterile water to each of the plates, with all visible growth removed. The liquid from each plate was combined in a single falcon tube (FB55959, Fisher, UK), vortexed for 30 seconds and then heated to 80°C for 10 minutes, to inactivate any vegetative cells. The spore suspensions were then centrifuged at 1500 rpm for 20 minutes and the supernatant discarded. The pellet was vortexed at 40 hertz for five seconds in 10ml of MRD, centrifuged and resuspended in another 10ml MRD; the wash process was repeated five times. The final pellet was

resuspended in 10ml of MRD and stored at 4°C until use. The presence of spores was confirmed by microscopy with heat-fixed slides stained using the differential Schaeffer-Fulton spore stain. The suspensions positive for spores by microscopy were then enumerated using BHIS/T agar plates and anaerobic incubation at 37°C for 48 hours. The investigation was repeated in triplicate on two separate occasions.

2.3.5.2 Broth culture methods

Pre-reduced BHIS agar plates were inoculated from a cryobead of *C. difficile* as previously described (section 2.2.2.1). After 24 hours anaerobic incubation at 37°C *C. difficile* colonies were transferred to 10ml aliquots of pre-reduced CMB in triplicate. After 24 hours anaerobic incubation at 37°C a stressor was applied to the broth to induce sporulation. The stressors investigated were:

- a. Alcohol stressor – 5ml of 70% ethanol was added to each broth culture, mixed well by agitation and inverted to re-suspend the settled cells. After 24 hours the spores were recovered.
- b. Aerobic stressor – the culture was moved to an aerobic incubator at 25°C for 96 hours.

Spores were then recovered by heating the broths to 80°C for 10 minutes, to inactivate any vegetative cells and then centrifuged at 1500 rpm for 20 minutes. The supernatant was discarded, the pellet was vortexed in 5ml of MRD and the six cultures were pooled into a single 50ml tube. The combined

suspension was centrifuged and washed in 10ml MRD five times to remove cell debris, broth and alcohol. The presence of spores was first confirmed by microscopy as previously described (section 2.2.5.1). The final suspensions were enumerated as described (section 2.2.4.3). The investigation was repeated in triplicate on two separate occasions.

2.3.5.3 Additional non-heat exposed stressor

The final method was used to produce a spore suspension which had not been pre-exposed to heat before testing thermotolerance (section 4.2.1), the method was developed by Alfa *et al.* (2013) and used with broth cultures. *C. difficile* broth cultures (6) were prepared in CMB as previously described (section 2.2.5.2). After 14 days anaerobic incubation at 37°C, cultures were pooled. The combined cultures were centrifuged at 1500 rpm for 20 minutes. The supernatant was discarded and the pellet was resuspended in 5ml of 70% ethanol for 20 minutes, to inactivate any vegetative cells. The suspension was then centrifuged at 1500 rpm for 20 minutes and washed five times in 5ml MRD to remove cell debris, alcohol and broth. The pellet was resuspended in 10ml MRD and homogenised for 20 seconds. Spore concentrations were confirmed and enumerated as previously described (section 2.2.5.2). The investigation was repeated in triplicate on two separate occasions. The method was then repeated using 20 pooled samples recovered into 5ml MRD, to achieve a higher

concentration of spores in the resulting suspension; this was repeated in duplicate on four separate occasions.

2.3.6 Storage test

C. difficile cell cultures in CMB were prepared as previously described (section 2.2.5.2). The broth-based aerobic stress method was used to induce sporulation (section 2.2.5.2 B). After recovering the suspension, the spores were enumerated and samples were taken on day 2, day 12 and day 30 of storage at 4°C. To enumerate, serial dilutions were made with duplicate 0.1ml samples inoculated onto BHIS/T. These were incubated anaerobically at 37°C for 48 hours. This was repeated in duplicate on eight occasions. In addition, spore suspensions were generated using the long incubation (14 days in CMB) method previously described (section 2.2.5.3). These suspensions were enumerated on day 0 and day 30 as previously described. This was repeated in triplicate on three separate occasions.

2.3.7 Statistical analyses

All statistical analyses were performed with IBM SPSS v. 22. The significance value for all tests was set at $p \leq 0.05$. The Shapiro-Wilk test for the normality of the distribution and Levene's test for equality of variance were used to

determine whether data could be analysed by Independent t-test and whether equal variance could be assumed. Where the assumptions of normal distribution were violated, the non-parametric Mann-Whitney *U* test was performed.

2.4 Results

2.4.1 Media assessment

The BHI media first investigated for the growth of *C. difficile* vegetative cells had varying success (section 2.2.2.1). The pre-reduced BHI agar alone was not able to support the growth of *C. difficile* vegetative cells. Once the BHI agar was supplemented with horse blood, cysteine and fructose, to form BHIS, the vegetative cells were able to grow (section 2.2.2.2).

With the addition of taurocholate sodium acid salt, forming BHIS/T, the *C. difficile* spores could consistently germinate, resume metabolic function and form visible colonies (Figure 2.1). In addition, this was further demonstrated by the growth of colonies from suspensions generated as part of the sporulation investigation (section 2.2.5.1).



Figure 2.1. *C. difficile* colonies on BHIS/T from germinated spores.

2.4.2 Identification of *C. difficile*

All identification methods provided evidence that the cultures were *C. difficile* (section 2.2.3). The BHIS plates streaked with broth culture and incubated aerobically at 37°C, showed no growth after 48 hours and 96 hours. Conversely, the BHIS plates incubated anaerobically, showed good growth of a single type of colony after 48 hours. The colonies had the distinctive manure smell, grey ground glass appearance and exhibited a yellow-green fluorescence

under long-wave UV light. The catalase test was negative, with the final latex agglutination test results providing definitive identification of *C. difficile*.

2.4.3 Growth curves

The growth of *C. difficile* vegetative cells in CMB, over a 48-hour period, showed the maximum cell concentration was reached around 8-10 hours of growth, this achieved $8.79 \log_{(10)} \text{ cfu/ml}$ (section 2.3.4.3). At 24 hours cell concentrations dropped to $8 \log_{(10)} \text{ cfu/ml}$ and at 48 hours cell numbers further reduced to $6.44 \log_{(10)} \text{ cfu/ml}$ (Figure 2.2).

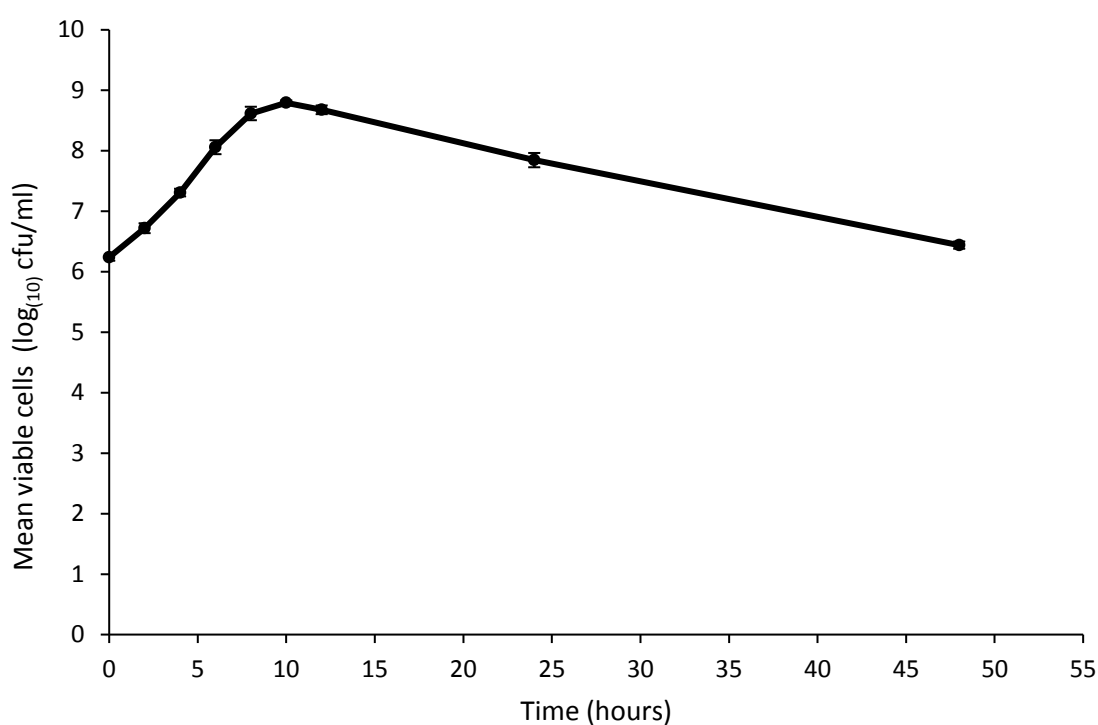


Figure 2.2. *C. difficile* cells grown in Cooked Meat Broth culture, over 48 hours at 37°C for 48 hours (mean \pm SE, $n=4$).

The spore enumeration curve showed an original mean inoculum of 2.48 $\log_{(10)}$ cfu/ml of spores, increasing to 3.88 $\log_{(10)}$ cfu/ml after 6 hours. This continued to increase to 5.88 $\log_{(10)}$ cfu/ml, 6.47 $\log_{(10)}$ cfu/ml and 6.45 $\log_{(10)}$ cfu/ml after 12, 24 and 48 hours respectively (Figure 2.3).

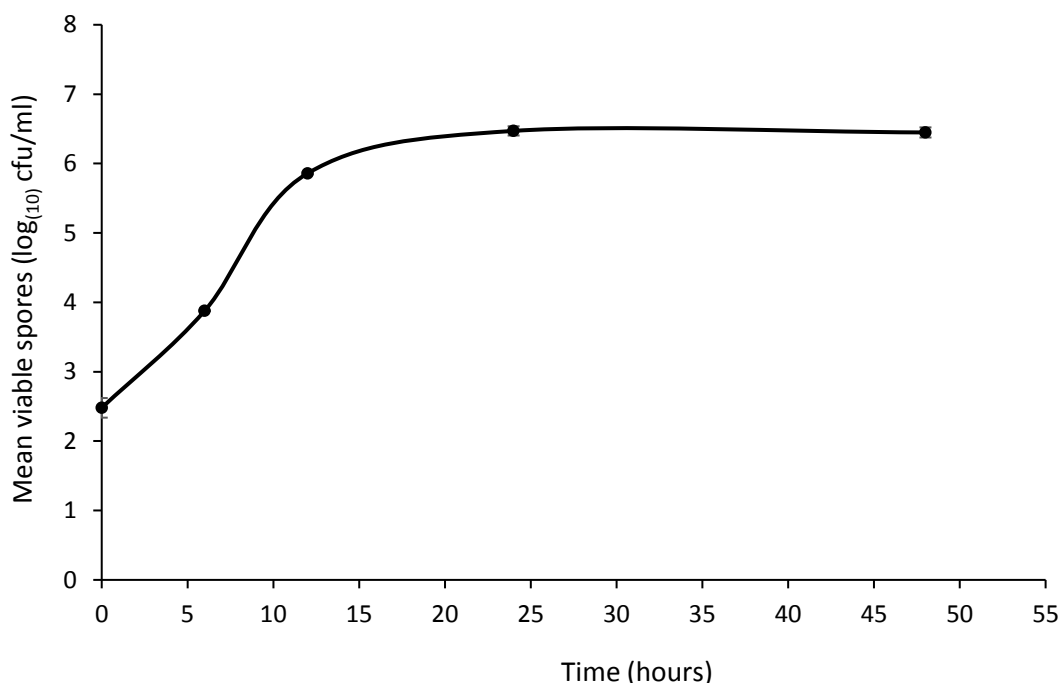


Figure 2.3. *C. difficile* spores sampled from CMB cultures (mean \pm SE, $n=4$).

2.4.4 Sporulation

The spore suspensions recovered from the agar plates, had very few spores visible by microscopy (section 2.3.5.1). The aerobic (method a) and long incubation stressor (five days on BHIS plates) (method d) plates produced very few spores (1 to 2 spores per microscopy slide). The alcohol stressor (method b) and temperature stressor (method c) plates also produced too few spores to

enumerate by germination on BHIS/T agar. The 30 minute alcohol exposure had no spores visible on the microscope slide, whereas 3 hour alcohol exposure had a range of 12-25 individual spores over an entire slide. The agar plate methods were difficult to compare as so few spores were present. The suspensions generated from agar plates were not enumerated and were discontinued.

The broth based methods had many more spores per slide when assessed by microscopy, so were enumerated by serial dilution and spread plating to obtain spore concentrations (Figure 2.4). Alcohol stress generated suspensions of $5.36 \log_{(10)} \text{ cfu/ml}$. The aerobic stress method, consistently generated spore suspensions with concentrations of $7.77 \log_{(10)} \text{ cfu/ml}$. Comparison by independent t-test confirmed that the alcohol stress method produced significantly lower spore concentrations than the aerobic stress method ($t= 49.768, p \leq 0.05$).

The long incubation (14 days in CMB) method, with inactivation of cells by alcohol, consistently generated spore suspensions of $6.51 \log_{(10)} \text{ cfu/ml}$. The concentration of spores generated was not high enough for the intended use, so the method was repeated using 20 pooled cultures in 5ml rather than 10ml MRD, which gave much higher concentrations of $8.06 \log_{(10)} \text{ cfu/ml}$ (Figure 2.4).

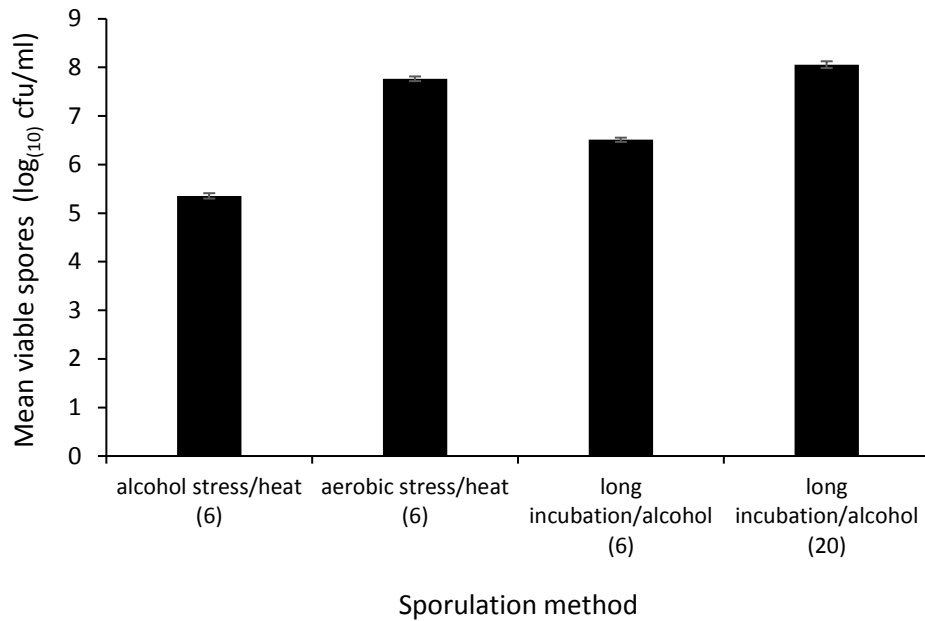


Figure 2.4. *C. difficile* spore concentration in suspensions generated by different methods using 6 pooled cultures (mean \pm SE, $n=6$,) and for 20 pooled cultures (mean \pm SE, $n=8$).

2.4.5 Storage test

The suspensions generated for the storage investigation were enumerated on the day of recovery, the spore concentration in the aerobic stress method (heat inactivation of cells) suspensions started with a mean concentration of 7.43 \log_{10} cfu/ml and on day 30 of storage at 4°C spore concentration was a mean of 7.48 \log_{10} cfu/ml (Figure 2.5). There was a slight increase overall in the mean spore concentration of 0.05 \log_{10} cfu/ml. The mean spore concentration for suspensions generated by long incubation (14 days in CMB) for the storage investigation was 6.48 \log_{10} cfu/ml and on day 30 the mean concentration was 6.49 \log_{10} cfu/ml. There was a slight increase of 0.01 \log_{10} cfu/ml (Figure 2.5).

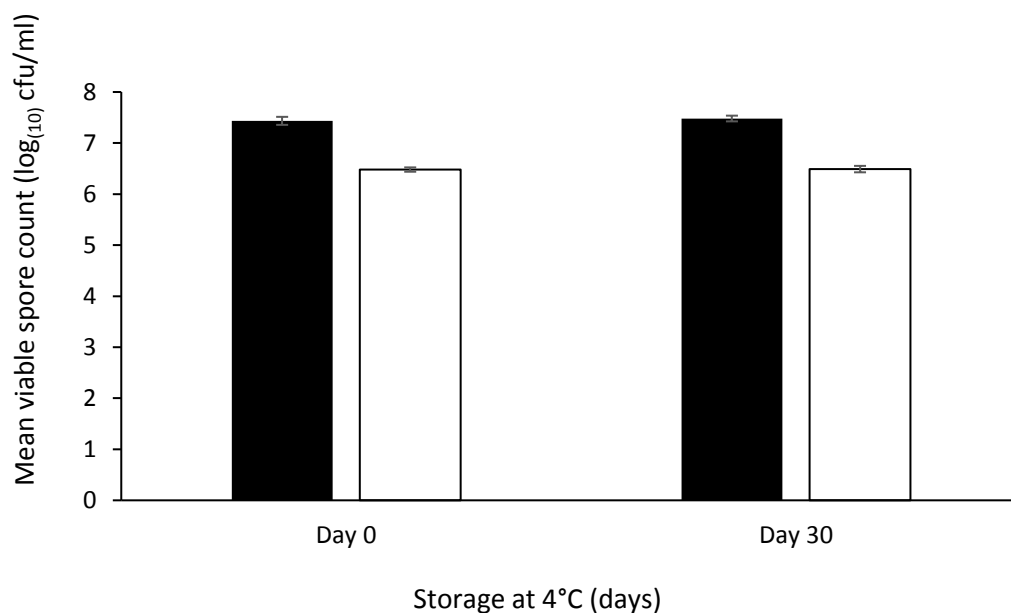


Figure 2.5. *C. difficile* spore concentration after storage (mean \pm SE). Spores generated by aerobic stress (■, $n=16$) or long incubation (□, $n=8$).

The enumeration data, from the aerobic stressed method (section 2.2.5.1. a.) were not significantly different from a normal distribution ($p>0.05$). There was no significant difference between spore concentrations on day 0 and day 30 ($t=-0.724$, $p>0.05$). The spore enumeration data from the long incubation (14 days in CMB) method (section 2.2.5.3), were significantly different from a normal distribution (day 0, $p\leq 0.05$). There was no significant difference between spore concentrations on day 0 and day 30 ($U= 134.50$, $p>0.05$).

2.5 Discussion

2.5.1 Growth curves

Efficient growth media for *C. difficile* cells was achieved with BHI base agar, supplemented with horse blood (Sorg and Dineen, 2009) (section 2.3.2); colonies were more visible for accurate counting and when viewing fluorescence for identification (section 2.3.3). Further supplementation with sodium taurocholate enabled the germination of spores and successful enumeration during the growth curves and sporulation investigations (sections 2.3.4 and 2.3.5).

The inoculated pre-reduced BHI broth, had no or very limited growth of *C. difficile* cells (section 2.3.4.2). After switching to pre-reduced CMB the cultures grew well, producing a thick cell layer of cells at the bottom of the universal and a very turbid culture when vortexed (section 2.3.4.3).

The *C. difficile* cells grew well in CMB, achieving a mean of 8.79 log₍₁₀₎ cfu/ml viable *C. difficile* cells after 12 hours anaerobic incubation at 37°C, with 8.00 and 6.44 log₍₁₀₎ cfu/ml by 24 and 48 hours, respectively (Figure 2.2). The maximum spore concentration was within the 5-7 log₍₁₀₎ cfu/ml range suggested by Burns *et al.* (2010), although the NCTC 11209 strain of *C. difficile* was not included in their study. The mean spore concentration from the growth curve at 24 and 48 hours was comparable to the 6.67 log₍₁₀₎ cfu/ml achieved by Pereira

et al. (2013), with the strain *C. difficile* 630Δerm at 48 hours. In addition, mean spore concentration was lower than Merrigan *et al.* (2010) who achieved mean spore concentration of 7.71 log₍₁₀₎ cfu/ml after 48 hours.

2.5.2 Sporulation

With the aim of producing suspensions with a high concentration of spores, broth cultures were incubated aerobically (after 24 hour anaerobic incubation) and then recovered using heat inactivation of vegetative cells (section 2.3.5.2). The extra stress on cells from aerobic incubation resulted in a mean spore concentration of 7.77 log₍₁₀₎ cfu/ml. In contrast, the use of alcohol to stress the culture resulted in a mean spore concentration to 5.36 log₍₁₀₎ cfu/ml (Figure 2.6). The significantly lower concentration of spores may have been due to the alcohol inactivating instead of stressing the vegetative cells ($p \leq 0.05$). The alcohol stress method (section 2.3.5.2 b) was discontinued due to low spore concentration and the aerobic stress method (section 2.3.5.2 a) was carried forward for further investigation. The aerobic stress method gave a comparable spore concentration to other published methods, for example, Hasan *et al.* (2011) used a long incubation (10-14 days on CDMN agar) to stress cells and had a final spore concentration of ~8 log₍₁₀₎ cfu/ml, although a pooled agar plate method was used for growing the cell culture. When the same method was followed in the present study, using long incubation (five days on BHIS agar) to

stress agar plate cultures, the resulting suspension had no spores visible by microscopy.

The method of spore production by long incubation (14 days in CMB), with alcohol inactivation of cells, was developed as a way of producing spore suspensions which were not pre-exposed to heat, for use further investigations assessing heat as a method of decontamination (section 2.3.5.3). The long incubation (14 days in CMB) method resulted in a mean spore concentration of $6.51 \log_{(10)} \text{ cfu/ml}$. Although from 6 pooled cultures, the mean was only marginally higher than the mean spore concentration for single CMB cultures seen in the growth curve ($6.47 \log_{(10)} \text{ cfu/ml}$), with heat inactivation of the cells (section 2.3.4.3). The pooling and recovery of 20 cultures to make 5 ml spore suspensions, increased the spore concentration to $8.06 \log_{(10)} \text{ cfu/ml}$; this was similar to Alfa *et al.* (2013) and Macdonald *et al.* (2016), both of which achieved $>8 \log_{(10)} \text{ cfu/ml}$ and $8.2 \log_{(10)} \text{ cfu/ml}$, utilizing long incubation (14 days) of agar plates and alcohol inactivation of cells. However, both previous studies used agar plates to culture the cells, not broth cultures, in contrast to the present study.

The aerobic stress method produced a greater spore concentration than the long incubation (14 days in CMB) method, but the methods differed in media type, the stressor and how cells were inactivated. This was mainly due to

the intended use of the suspensions after recovery. For example, the aerobic stress and heat inactivation method was a preliminary method to obtain spores for testing in non-heat related investigations assessing recovery of spores from NHS (100% cotton) sheet material. The long incubation with alcohol inactivation of cells method was developed by Alfa *et al.* (2013), to be used in the study of spore thermotolerance in bed pan washer decontamination cycles. Inactivating vegetative cells using alcohol prevented pre-exposing the spores to high temperatures and made it unlikely that they would accumulate damage. Given the complete absence of spores in the agar plate methods tried, the long incubation (14 days in CMB) method was a reasonable modification. Although Hasan *et al.* (2011) reported long incubation of CABA plates to be the most efficient at inducing sporulation, this was not the case in the present study. The poor performance of the agar methods investigated in the present study, as compared to published methods, may be due to the use of BHIS/T instead of pre-made commercial CABA plates, or the use of a different strain of *C. difficile*.

2.5.3 Storage

The aerobic stress and long incubation (14 days in CMB) methods produced spore suspensions which showed no statistically significant decline in viable spore concentration over 30 days. For the aerobic stress method, there was a small but statistically insignificant increase in mean viable spore concentration

over the 30 day period. The stability of spores suspensions during storage was supported by Freeman and Wilcox (2003), who found that spores stored in faecal emulsion, showed no significant decline in viable non-heated spore concentration over 56 days aerobic storage at 4°C, with either single or repeated sampling. Cells were also inactivated by alcohol and enumerated in agar containing taurocholate. This contrasts with Rodriguez-Palacios (2011), who found there was a decline of 0.28 log₍₁₀₎ cfu/ml in the spore concentration of non-heated spore suspensions stored for 18 days aerobic storage at 4°C. The media was supplemented with taurocholate and the vegetative cells were inactivated by alcohol. Interestingly, there was no decline in spore concentration in heated suspensions, over the 18 day period. The authors suggest this may be due to superdormancy which is common in *Bacillus* spp. (Ghosh and Setlow, 2009).

Weese *et al.* (2000) similarly found that the number of equine faecal samples with *C. difficile* present reduced over 72 hours aerobic storage at 4°C, down to 29% of samples testing positive for *C. difficile*. However, the media used didn't appear to contain taurocholate and supplementation was not reported. Therefore, the decline in positive *C. difficile* faecal emulsions, stored aerobically and the stability when stored anaerobically are likely a result of the cells inability to tolerate oxygen and is unlikely to represent the effect of storage on spore viability.

2.6 Conclusions

The aerobic stress of broth cultures, was superior to the alcohol stress when producing spores for early non-heat related investigations. The long incubation (14 days in CMB) method was developed for subsequent heat-treatment investigations and when multiple cultures were pooled, gave an acceptable mean spore concentration of $8.06 \log_{(10)} \text{ cfu/ml}$. Storage was not shown to affect viability of spores, with no reduction in spore concentration in non-heated or heated suspensions over 30 days. Differences in media or *C. difficile* strain may account for the previously reported effect of storage and reducing spore concentration in non-heated suspensions. In contrast, the literature supports the findings that heated spore suspensions are not affected by storage and exhibit no loss of viability.

**3 Investigation of the efficacy of the UK
Department of Health infected laundry policy;
Health Technical Memorandum 01- 04**

3.1 Chapter abstract

The laundry policy, HTM 01-04, sets the thermal disinfection temperature for an infected linen wash: 71°C for ≥ 3 minutes (+ mixing time), or 65°C for ≥ 10 minutes (+ mixing time). The disinfection cycle should be able to pass microbiological standards e.g. no bacteria on previously sterile de-sized textiles, $>5 \log_{(10)}$ reduction of a thermotolerant species of bacteria and <100 cfu with no pathogenic bacteria. The aim of the study was to quantify the survival of *C. difficile* spores on cotton during a simulated WE cycle and a WE cycle *in situ* at a commercial laundry. A survey was conducted to explore how care facilities implement HTM 01-04. The simulated WE cycle, with an industrial detergent, demonstrated survival of two strains NCTC 11209 (0-4 cfu/25cm²) and ribotype 001/072 (0-9 cfu/25cm²). Before washing in the commercial WE the average spore load was 51 cfu/25cm² and after washing, drying and finishing was 33 cfu/25cm². Both the simulated and *in situ* linen failed the microbiological standards. The method of agitation used to recover spores from cotton, was shown to be important; vortexing (4.48 $\log_{(10)}$ cfu/ml) recovered more spores than stomaching (4.20 $\log_{(10)}$ cfu/ml) in the presence of soiling. The survey demonstrated that some care facilities may not be meeting minimum requirements e.g. not using alginate bags for infected linen or not having annual validation of the disinfection cycle. Commercial WE cycles may be exposing patients to low levels of *C. difficile* spores.

3.2 Introduction

Textile items are common throughout the healthcare environment and include curtains, carpets, towels, uniforms, bed linen and equipment such as hoists and pressure cuffs (Sehulster and Chin, 2004; Department of Health, 2016a). There is the potential for these textiles to become vehicles for transmission of infectious agents; extensive evidence demonstrates common contamination of uniforms, white coats and bed linen (Barrie *et al.*, 1994; Perry *et al.*, 2001; Patel *et al.*, 2006; Wilson *et al.*, 2007; Treakle *et al.*, 2009; Fijan and Turk, 2012; Gupta *et al.*, 2016).

Perry *et al.* (2001) sampled nursing staff uniforms at the start and end of duty and found frequent contamination with MRSA, Vancomycin-resistant *Enterococcus* (VRE) and *C. difficile* spores. Before duty VRE was found on 12/56 uniforms and MRSA on 7/57, with contamination levels varied from 1-100 cfu. After duty the number of positive samples increased, with VRE isolated on 22/57 uniforms and MRSA on 8/57 uniforms. *C. difficile* spore contamination was around 1-10 cfu, with 7/56 HCW uniforms positive before duty and 11/56 positive after duty (Perry *et al.*, 2001). Interestingly, several of the uniforms which had been positive at the start of duty were found to be negative at the end. This was found on four occasions with MRSA, five with VRE and five with *C. difficile*. Given the longevity and high resistance of *C. difficile* spores to decontamination, it could be that the spores were transferred to other surfaces,

HCW or patients. Treacle *et al.* (2009) demonstrated common *S. aureus* contamination of white coats, at around 22.8% (34/149) and MRSA contamination around 4% (6/149).

All patients have a right to expect care to be provided in a ‘...clean and safe environment that is fit for purpose, based on national best practice’ (Johnson, 2008). Therefore, an effective textile laundering service is required, with most UK hospitals using commercial facilities to launder textiles and/or provide linen rental services for items such as bed sheets (TSA, 2015). The processing of hospital linen is covered by HTM 01-04 Decontamination of Linen for Health and Social Care (2016). HTM 01-04 sets the conditions for both commercial and on-site laundering of used linen and infected linen. The four volumes within HTM 01-04 cover onsite laundering within adult social care facilities, overall management of the laundry process, the engineering, equipment and requirements for validation and a guide to using biocontamination control (Department of Health, 2016a; Department of Health, 2016b; Department of Health, 2016c; Department of Health, 2016d; British Standards Institution, 2016).

The main purposes of the laundering process are to provide visibly clean, microbiologically safe textiles, which are of the appropriate material, to protect vulnerable patients from infection (Fijan *et al.*, 2005; Fijan *et al.*, 2006;

Department of Health, 2016a). The key components to providing an effective laundry process are dilution, detergent, agitation and thermal or chemical decontamination to inactivate microorganisms (Wilson *et al.*, 2007; Department of Health, 1995; Department of Health, 2013; Department of Health, 2016; Bockmühl, 2017).

The management and provision volume of HTM 01-04, gives guidance on the listing of critical control points necessary to ensure laundries are meeting the Essential Quality Requirements (EQR) (Department of Health, 2016c). Validation requirements of the critical control points are covered in the HTM 01-04 engineering and validation volume, these include ensuring minimum temperatures are met and the routine annual checking of microbiological quality of the finished products by semi-permeable dose strip test with thermotolerant *Enterococcus* spp. (Department of Health, 2016a).

The validation volume suggests additional checks may be warranted to assess the efficacy of the laundry process to decontaminate thermotolerant spores, particularly *B. cereus*, although a mandatory method is not reported. The linen operator is advised that a potential method is described within 'BS EN 14698-1: Clean Rooms and Associated Controlled Environments, Biocontamination Control: General Principles and Methods (British Standards Institution, 2003)'. The method describes inoculating pieces of material, then

processing these pieces with regular linen and recovering viable organisms using agitation in recovery medium, to recover cells and spores for assessing microbiological quality. There are industrial standards, which are primarily for the testing of antimicrobial coating on textiles, which can provide methods for the recovering of viable organisms. For example, the Japanese Industrial Standard (JIS) 1902:2002 and BS EN 14698-1:2003 which advocates 'agitation' without specifying the specific method or length of agitation time.

3.2.1 The UK healthcare laundry policy

3.2.1.1 Development of the UK healthcare laundry policy

The healthcare laundry policy, HSG (95) 18 Hospital Arrangements for Used and Infected Linen was in force between 1995 and 2013 (Department of Health, 1995). Linen was classified as one of three types: Used (soiled and fouled), Infected or Heat labile. For the purposes of this study, the focus was on the infected linen pathway. In brief, the infected linen route specified that all linen classified as 'Infected' must be put immediately into water-soluble bags (alginate bags) and then into non-permeable bags. The linen must be processed with a thermal disinfection cycle at either 65°C for ≥ 10 minutes or at 71°C for ≥ 3 minutes, with mixing time added on to ensure all fabric has been exposed for the minimum time stated. The mixing time was dependent on the weight of fabric to water ratio, so at <0.056 Kg/l the mixing time was 4 minutes and at

>0.056 Kg/l mixing time was 8 minutes. Infected linen must not be hand sorted before processing, and must not enter a CTW before being decontaminated, due to health and safety concerns if there are blockages in parts of the machine which can't be thermally disinfected. Infected linen must be washed in a WE and fouled linen must be processed with a sluice cycle to remove heavy solid soil. In addition to the cycle parameters, there were conditions that must be met in terms of separation of dirty linen entering the laundry and clean processed linen waiting to be transported to the end user.

The HSG (95) 18 policy, also had guidance for 'small facilities', presumably adult social care sites, primarily that the same linen classification system, wash cycle and safety conditions were necessary. In 2013 the policy was updated, with a focus on spore bearing microorganisms, mainly due to growing evidence of *B. cereus* spore contamination of laundered linen (considered fully in section 3.1.2.) (Barrie *et al.*, 1994; Dohmae *et al.*, 2008; Sasahara *et al.*, 2011). In brief, when high levels of spores up to 5 log₍₁₀₎ cfu/g fabric, are present on linen entering a CTW, the chemo-thermal conditions may not be sufficient for adequate decontamination (Barrie *et al.*, 1994; Dohmae *et al.*, 2008; Sasahara *et al.*, 2011). Where sporulation occurs on linen stored in bags during warm weather, chemo-thermal disinfection processes may be unable to decontaminate the linen.

The updated document was the Choice Framework for Policy and Procedures 01-04: Decontamination of Linen for Health and Social Care (CFPP 01-04). The update had a much broader scope to address the variation in laundry service providers. The thermal disinfection parameters remained the same, but chemo-thermal, chemical or alternative disinfection cycles e.g. Ozone washers, were addressed (Department of Health, 2013). The use of chemical or alternative disinfection was required to be at least as effective as thermal disinfection alone. In addition, there was a section more fully describing the social care laundry provision and the engineering and validation requirements at any healthcare laundry service provider. There was an information box mentioning the possibility of 'low levels' of *C. difficile* spore survival, but no specific requirement to test, quantify survival or validate wash cycles for *C. difficile* spores. It seems that this was regarded as unimportant, simply because there was no causal link between *C. difficile* surviving on linen and an active CDI. The contribution of surviving spores on linen to asymptomatic carriage of *C. difficile* has been not been considered, particularly within care facility settings.

The most recent update is the Health Technical Memorandum (HTM) 01-04 Decontamination of Linen for Health and Social Care (2016). The update was essentially a renaming of the CFPP 01-04 policy document to match other health technical manuals. As well as updating the name, there was the addition of a section addressing those laundries which have met BS EN 14605

Biocontamination control and are independently accredited. The volume discusses the equivalencies between BS EN 14605 certification and meeting the minimum standards and best practice of the HTM 01-04 policy.

In summary, the thermal disinfection conditions have remained the same throughout the updates of the healthcare laundry policy, from HSG (95) 18 to CFPP 01-04 and in the most recent HTM 01-04. The updates have attempted to address changing technologies and the different ways of meeting the minimum requirements, while relaxing the requirements for care facilities not using the infected linen pathway. The first mention of issues with the reduction of thermotolerant spores from *B. cereus* and *C. difficile* was reported in CFPP 01-04 (2013) and continues in HTM 01-04 (2016). Quantifying *B. cereus* spore contamination was suggested as an annual test, without specific methods for doing so, and there was no requirement for evaluating *C. difficile* spore contamination.

3.2.1.2 HTM 01-04 and the infected linen pathway.

The processing of used linen generated within the hospital environment is now subject to HTM 01-04 and so must be sorted at the bedside into 'Used (soiled or fouled) linen' and 'Infected linen'. All infected linen must be placed into a water-soluble bag, then into a non-permeable outer bag labelled 'Infected linen'. The wash cycle must have a decontamination process such as a minimum time and temperature cycle or chemical-based disinfection as previously described

(section 3.1.1.1). The wash process must meet the microbiological test validation; there must be no bacteria recovered from a previously sterile de-sized textile (Department of Health, 2016a). For meeting the best-practice, additional microbiological assessments should be performed annually using thermotolerant bacteria such as *E. faecalis* or *E. faecium*, where a $>5 \log_{(10)}$ reduction must be achieved, when tested before drying and finishing.

The annual assessment of efficacy against by *B. cereus* spores is advised, although there is no mandatory method reported. Reference is made to Annex E of BS EN 14698-1 Clean Rooms and Associated Controlled Environments, Biocontamination Control: General Principles and Methods (British Standards Institution, 2003). Annex E describes a destructive sampling and agitation method, with the quantification of target organisms by filtration of suspensions eluted from the fabric (British Standards Institution, 2003; Department of Health, 2016a). The duration and level of effort of the chosen agitation method is not reported, therefore, the findings cannot be compared across laboratories that are responsible for validating the disinfection cycles of healthcare laundries.

3.2.1.3 Healthcare laundry in the adult social care sector

As the healthcare laundry policy has been updated, the requirements for social care have developed toward a more relaxed approach for facilities which do not deal with infected linen on a regular basis. The current policy, HTM 01-04,

assumes that most adult care providers will be using a standard process most of the time for stable and healthy adult patients. For the standard laundry, the EQR are: a domestic washing machine in good working order with regularly servicing; training in the use of the machine and dosing of detergents for operatives; segregated clean and dirty areas; room to sluice soiled linen; and prevention of infection for staff and service-users. In the case of known infectious linen an enhanced route is necessary, which is the equivalent of the infected linen pathway previously described (section 3.1.1.2).

In brief, the enhanced route requires an industrial machine, capable of achieving the thermal disinfection conditions previously described. As in the main volume of HTM 01-04, a chemical disinfection system may be used which meets or exceeds the effectiveness of thermal disinfection. Whichever disinfection method is chosen, the machine must be professionally installed and maintained, with annual validation of the disinfection stage and handling of infected linen must be minimized, through the use of alginate bags. The processing of linen may be outsourced to a commercial healthcare laundry, which meets the minimum requirements of HTM 01-04, or by a linen rental contract. The responsibility lies with each care facility to ensure they are using the enhanced process where necessary or outsourcing to a commercial laundry which can meet the same requirements.

3.2.2 *C. difficile* and *B. cereus* spores; links to healthcare textiles and the healthcare laundry policy

There are few studies exploring the thermal and chemo-thermal disinfection measures used in healthcare laundry and the level of survival of *C. difficile* spores. Hellickson & Owens (2007) found viable spores and cross-contamination during laboratory-based simulated washes. Sterile cotton swatches and cotton swatches inoculated with $\sim 4 \log_{(10)}$ *C. difficile* (ATCC 9689) spores were exposed to minimum temperatures, commonly used detergents and a set of additives: 50 ppm chlorine, 54 ppm peracid or 100 ppm peroxide for chemo-thermal decontamination. Both sterile and inoculated swatches were sampled post-wash and were contaminated with viable spores in all treatments. *C. difficile* spores were recovered from the swatches by incubation in BHI broth and recorded as presence or absence of growth, with no identification or quantification. This study would have been more persuasive if it had attempted to quantify spore survival and confirm that the growth in media was indeed germinated *C. difficile* spores which had been previously inoculated onto the swatches.

Later work by Lakdawalla *et al.* (2011), recovered *C. difficile* spores from labelled linens washed in a CTW cycle which met the thermal disinfection minimum standard of 71°C for ≥ 3 minutes. A total of $10^1 - 10^3$ cfu/100cm² was found on linen washed as non-infected. The ribotypes of the isolates from the

linen after washing matched the isolates from the CDAD patients who had provided the linen, except one sheet which had an additional ribotype. The original spore contamination was not investigated. Positive samples were isolated from sheets washed as standard linen which is of concern, as HSG (95) 18 in force in 2011, clearly stated that infected linen must not be processed in a CTW. It is not clear why the infected sheets were not washed in a WE, either as a standalone healthcare compliant cycle or a pre-wash stage before washing in a CTW.

More recently, *C. difficile* spores have been recovered at 6 log₍₁₀₎ cfu/swatch and 5 log₍₁₀₎ cfu/swatch on inoculated swatches from an ambulance uniform, washed at 30°C and 60°C respectively (Mackay *et al.*, 2017). The 60°C wash was able to reduce the spore load by 1 log₍₁₀₎ cfu/swatch compared to the 6 log₍₁₀₎ cfu/swatch recovered from inoculated but unwashed swatches. Furthermore, spores survived on a single reusable mop strand which was washed at 95°C. There may have been viable spores on other strands, as the limit of detection was particularly high (>3.4 log₍₁₀₎ cfu/ml) due to use of the Miles and Misra technique, rather than the spread plate method.

There is more evidence regarding *B. cereus* spores, where not only survival of spores through the wash have been shown, but surviving spores have been linked to active infection in patients exposed to the laundered items. In a *B. cereus* meningitis outbreak investigation carried out by Barrie *et al.* (1994), it

was demonstrated that *B. cereus* spores were able to survive on linen washed in a CTW. The wash conditions included a phenolic disinfectant and CTW wash compartments which reached 80°C. By sampling of water entering and leaving the CTW pre-wash compartment, it was calculated that up to 5 log₍₁₀₎ cfu/g fabric were entering the CTW wash cycle. It was concluded that the number of spores was too great for the chemo-thermal decontamination to be effective; sampling of stored used linen showed high proliferation of spores in warm storage conditions.

In another outbreak investigation, Dohmae *et al.* (2008) recovered *B. cereus* spores from hospital towels (6 log₍₁₀₎ cfu/towel), laundered at an onsite hospital laundry. The spores were closely related to patient isolates, in wards with a *B. cereus* outbreak (Dohmae *et al.*, 2008). The outbreak ended after the towels were decontaminated at an external laundry using sodium hypochlorite. Nosocomial bacteremia caused by *B. cereus* spores from contaminated linen was also demonstrated by Sasahara *et al.* (2011). There was a range of 2-3 log₍₁₀₎ cfu/cm² *B. cereus* spores recovered from laundered bed sheets, while 4-5 log₍₁₀₎ cfu/cm² was recovered from towels. The isolates recovered from bed linen, towels and patients were highly related; it was shown that transmission occurred from bed linen to patient via intra-venous catheter infection.

C. difficile is anaerobic, so spores are unlikely to proliferate during linen storage in warm conditions, but there may be between 5-7 log₍₁₀₎ cfu/g faeces

evacuated onto bed linen by symptomatic and, perhaps more importantly, asymptomatic patients (Riggs *et al.*, 2007). Although the levels of *C. difficile* spores actually entering the wash have not yet been estimated in the published literature. This would be an important first step in understanding the relationship between *C. difficile* spores and whether they may survive the laundry process.

C. difficile spores exhibit *in vitro* resistance to temperatures much higher and for much longer than the healthcare laundry policies require, e.g. 90°C for up to 10 minutes (Rodriguez-Palacios *et al.*, 2010; Rodriguez-Palacios and LeJeune, 2011). However, Orr *et al.* (2002) demonstrated that with enterococci, *in vitro* thermotolerance does not necessarily equate to survival of a healthcare wash cycle which meets the minimum thermal disinfection requirements as previously discussed (section 1.2.2). The previous studies exploring *C. difficile* spore survival of thermal disinfection cycles, have not quantified the spore load before and after the wash and has therefore been unable to calculate the reduction effect of a CTW cycle. Alternatively, evidence of survival of *C. difficile* spores has been part of a simulated wash which has only reported the presence or absence of spores.

3.2.3 Agitation methods for removal of cells or spores from textiles

Several test standards were consulted to define methods for the removal of spores from textiles in order to determine contamination levels. The first was BS EN 14698-1: Cleanrooms and associated controlled environments; Biocontamination control, General principles and methods (British Standards Institution, 2003). The BS EN 14698-1 test protocol involves destructive sampling of a washed, dried and finished sheet. The agitation method suggested was stomaching, followed by membrane filtration of the resulting suspension to quantify the spores recovered. Other methods were sought to compare with the stomaching method, including the JIS L1902:2002 antimicrobial finish testing standard, which suggests either hand shaking over 30cm (30 seconds), or vortexing for 5 seconds, five times. Also considered was Jenkins and Sherbourne (2005) which assessed the recovery of bacterial cells using vortexing (1 minute), sonication (30 seconds), rotary shaking (10 minutes) and glass rod agitation for (20 seconds).

3.2.4 In summary

The UK healthcare laundry policy HTM 01-04, provides a framework around which a commercial laundry or care facilities can design and operate an infected linen laundry process. How each laundry site meets the minimum thermal or chemical disinfection requirements can be unique to the site, based on

machines available, sorting system in place, physical space and the availability of detergents. In addition, the exact wash cycle including the amount of water, number of rinses, temperature of rinses and the use of pre-wash or sluice cycles can vary. The validation volume of HTM 01-04 makes an attempt to offer a method of comparison between one site process and another. This was developed to measure efficacy to inactivate thermotolerant bacterial cells. Furthermore, it suggests a test for thermotolerant *B. cereus* spores in the appropriate season, but without a compulsory method. Although *C. difficile* spores are considered in HTM 01-04, there are no specific requirements regarding testing or validation to ensure decontamination of *C. difficile* from infected linen.

There are a few published *in vitro* studies which provide preliminary evidence that *C. difficile* spores can survive parts of the laundry cycle as described in HTM 01-04, due in part to high thermotolerance as proliferation during storage is unlikely. Certainly, other spore formers have been shown to survive, when there are high concentrations of spores entering the laundry cycle, although the studies assessing spore survival are now outdated. There are a variety of sources of information regarding how to recover spores from swatches such as BS EN 14698-1 and JIS L1902:2002 Testing for Antibacterial Activity and Efficacy on Textile Products. There are also a number studies which use various methods of agitation to remove bacterial cells and spores, these

methods include vortexing, stomaching, hand shaking, sonication and rotary shaking.

3.2.5 Scope of the work

3.2.5.1 Aim

The aim of this investigation was to quantify the survival of *C. difficile* spores on NHS (100% cotton) bed sheets after washing under chemo-thermal disinfection conditions which meet the NHS Policy HTM 01-04 (2016).

3.2.5.2 Objectives

1. To determine a reliable, repeatable and efficient method for maximizing recovery of *C. difficile* spores from NHS (100% cotton) sheet swatches.
2. To assess the effect of soiling on the recovery of *C. difficile* spores from NHS (100% cotton) sheet swatches.
3. To quantify the survival of *C. difficile* spores on NHS (100% cotton) sheet swatches in simulated washes using the thermal and chemo-thermal conditions set out in the NHS laundry policy HTM 01-04.
4. To visualize NHS (100% cotton) sheet material with and without simulated soiling.
5. To quantify the survival of *C. difficile* spores on naturally contaminated experimental (100% cotton) sheets in an industrial WE

cycle, with drying and finishing at a commercial laundry conforming to the minimum conditions of HTM 01-04 (2016).

6. To compare how social care facilities implement the enhanced process wash conditions to conform to the social care volume of HTM 01-04 (2016).

3.3 Methods

3.3.1 Materials

There were two types of bed sheet which were supplied for the study: standard NHS (100% cotton) bed sheets supplied by the collaborating commercial laundry and blue experimental (100% cotton) bed sheets which were chosen for identification on the ward and at the laundry, purchased from the linen supplier used by the commercial laundry (B100, Mitre, UK). The material, finish and weave of the experimental sheets were all identical to standard NHS (100% cotton) sheets. The NHS (100% cotton) sheets were used to compare the preliminary methods of recovering spores (section 3.2.2), in assessing the effect of soiling on the recovery of spores (section 3.2.3) and in running simulations of a healthcare wash cycle (section 3.2.4). The blue experimental (100% cotton) sheets were used in assessing the effect of soiling on the recovery of spores

(section 3.2.3) and for assessing healthcare laundry policy using linens naturally contaminated with *C. difficile* spores (section 3.2.5).

3.3.2 Recovering *C. difficile* spores from air-dried cotton swatches

Swatches (25cm²) were cut from the central regions of the NHS (100% cotton) sheets and autoclaved before use. The swatches were then inoculated with 0.1ml of a 5 log₍₁₀₎ cfu/ml *C. difficile* NCTC 11209 spore suspension solution, generated using the aerobic stress method (section 2.2.5.2 B). Swatches were air-dried for 18 hours and either transferred to a stomacher bag (Mix3156, SLS, UK), glass beaker or falcon tube containing 30mls MRD and treated with one of five treatments:

- A. Stomaching – The swatch was placed in the stomacher bag and stomached in a paddle blender (LM40, Seward, UK) for 30 seconds or 1 minute at normal or high level setting.
- B. Vortexing – The swatch was placed in a sterile falcon tube, then vortexed on high at 40 hertz for five bursts of 5 seconds.
- C. Glass bead agitation – The swatch was placed in a sterile beaker, with 10 sterile glass beads (GS/2, SLS, UK) on top of the swatch and rotated for 1 minute at 150 rpm in a rotary shaker bath at 25°C (JBAqua 18, Grant, UK).

- D. Hand shaking- The swatch was placed in a sterile tube, then shook back and forth over a 30cm measured distance for 1 minute.
- E. Sonication of tubes on high for 1 minute in a sonic bath (XUBA3, Grant, UK).

After each treatment the swatches were left in MRD for 5 minutes, then removed and discarded. The resulting suspensions were then serially diluted and spread-plated in duplicate on BHIS/T agar plates. An enumeration control was performed to quantify the original inoculum by serial dilution of the test suspensions, followed by duplicate spread plating of 0.1ml samples onto BHIS/T. All agar plates were incubated anaerobically at 37°C for 48 hours. The investigation was repeated in duplicate on two separate occasions.

3.3.3 Soiling effect on *C. difficile* spore recovery from swatches

The vortex and stomach methods (section 3.2.1 A and B) were used to assess the effects of soiling on the recovery of spores from the NHS (100% cotton) sheet swatches. Bovine Serum Albumin (BSA), was used to simulate soiling, in 'clean' (0.3g/L) and 'dirty' (3g/L) conditions as per BS EN 13704 Chemical disinfectants – Quantitative suspension test for the evaluation of sporicidal activity of chemical disinfectant used in food, industrial, domestic and institutional areas –Test method and requirements (phase 2, step 1). Vortexing

was also used to assess the recovery of viable spores from the blue experimental (100% cotton) sheets in 'clean' and 'dirty' conditions.

The BSA solutions were filter sterilised, stored at 4°C and used within one month. The solutions were made up at 30g/L BSA or 3g/L BSA for dilution with the spore suspensions. The sterile swatches were inoculated with a 0.1ml of a $6 \log_{10}$ cfu/ml spore test solution and air-dried for 18 hours. The swatches were transferred to either a stomacher bag or falcon tube with 10ml MRD, then stomached on high for 1 minute or vortexed as previously described (method 3.2.1. A and B). Swatches were left in the MRD for 5 minutes after treatment and then removed and discarded; 0.1ml of the supernatant was plated onto BHIS/T agar. In addition, the vortexing method (section 3.2.1.B) was applied to a set of experimental (100% cotton) sheet swatches (25cm²). An enumeration control was performed to quantify the original inoculum by serial dilution of the spore test suspensions, followed by duplicate spread plating of 0.1ml samples onto BHIS/T. All agar plates were incubated anaerobically at 37°C for 48 hours. The investigation was repeated in duplicate on two separate occasions.

3.3.4 Quantification of the survival of *C. difficile* spores in simulated washes

A healthcare wash cycle programme was developed to assess heat, chemical and physical removal of *C. difficile* spores from NHS (100% cotton) sheet swatches. In preparation for the testing of thermal disinfection, the spore suspensions were generated, as previously described (section 2.2.5.3.). The industrial grade washing machine, a Schulthess Topline 6606 6.5kg, was programmed by the industrial detergent supplier to a test-specific programme. The programme was designed to meet the policy minimum requirement of 71°C for ≥3 minutes (+8 minutes mixing time). The water and detergent levels were also set by the detergent supplier, Washing Systems (Table 3.1); the detergent was supplied under the condition of confidentiality.

Sets of four swatches of NHS (100% cotton) sheets were labelled with an 'I' (Inoculated) or an 'S' (Sterile), individually attached to a safety pin and sterilised. The inoculated swatches had 0.1ml of an 8 log₍₁₀₎ cfu/ml spore test suspension (*C. difficile* NCTC 11209) containing 3g/L BSA, placed on them and were air-dried for 18 hours.

Table 3.1. Experimental NHS healthcare wash cycle meeting minimum temperature requirement for thermal disinfection.

Stage	Process	Water (L)	Temp (°C)	Time (min)
1	Detergent (total 120ml)	21.5	40	2 min
2	Thermal disinfection	21.5	75	10 min
3	Drain	-	-	-
4	Bleach (50ml)	29.5	60	5
5	Drain	-	-	-
6	Rinse	22	Cold input	2
7	Drain	-	-	-
8	Sour rinse with peracetic acid (50ml)	26	Cold input	2
9	Drain	-	-	-
10	Spin	-	-	2

Immediately before washing the inoculated swatches were attached to a single full size NHS (100% cotton) sheet, along with a set of four sterile swatches. The drum load was made up to maximum capacity (6.5kg) with 10

sterile NHS (100% cotton) sheets. A data logger (Ibutton, Measurement systems Ltd, UK) was used to take temperature readings every 5 seconds to verify that the minimum temperature of 71°C had been reached for a minimum of 3 minutes with an additional 8 minutes mixing time (11 minutes minimum).

After the full wash cycle was completed, each swatch was immersed in 30ml of MRD and vortexed (method 3.2.1.B). Where detergent was used the entire recovered suspension was immediately vacuum filtered using a sterile filter unit and black 0.45µm membrane filter (fil123, SLS, UK). For control cycles, 0.1 ml and 1 ml samples were filtered. Each sample was filtered with 50ml of SDW and then rinsed with a further 150ml SDW. After filtration, the membrane was immediately placed on a BHIS/T agar plate, with care to not trap air underneath, and incubated at 37°C for 48 hours. A 95°C wash cycle was completed after each test cycle to decontaminate and rinse the drum between cycles; the drum was swabbed using sterile swabs, pre-moistened with MRD, to check spore contamination of the machine. A daily healthcare cycle, run without linen is a requirement of HTM 01-04.

The wash cycle was repeated once a day on four occasions, with four independently generated spore suspensions using the long incubation (14 days in CMB) method (section 2.2.5.3). Furthermore, four independent control washes were repeated without the detergent system to provide a control by which the thermal disinfection alone could be assessed. The four independent

wash cycles and four control cycles were then repeated with spore suspensions of the *C. difficile* strain ribotype 001/072, recovered during the investigation described in section 3.2.5.

After all cycles were complete the washing machine was terminally decontaminated with Distel non-foaming High Level Medical Disinfectant, used in the washing machine on a 95°C wash cycle to access all areas of the washing machine. There was a final decontamination of the inner drum using Virkon 1%, which was left for 30 minutes and followed by a water rinse cycle to prevent corrosion. After decontamination, the drum of the washing machine was swabbed using sterile swabs, pre-moistened with MRD, to check spore contamination of the machine.

3.3.5 SEM of NHS (100% cotton) sheet swatches

For a visual comparison of roughness, material swatches were prepared and fixed from new NHS (100% cotton) sheet material and older NHS (100% cotton) sheet material donated by the collaborating commercial laundry (section 3.2.6) which was representative of a clean sheet in use, within the linen rental system. The old and new material swatches were prepared with and without soil (0.1ml of 3g/L BSA). In addition, a single cotton swatch was taken from a simulated wash (section 3.2.4) which failed the validation requirement for minimum time

at 71°C (e.g. was at 71°C for 5 minutes instead of 13 minutes for validated cycles).

The swatches were fixed and prepared for viewing by SEM using 2% glutaraldehyde (G7526, Sigma-Aldrich, UK) in phosphate buffer. Phosphate buffer (1M) was made with 11.99g/L monosodium phosphate, 14.19g/L disodium phosphate and adjusted to pH 7.4 using sodium hydroxide solution.

Samples of 1cm² were taken from the centre of the 25cm² swatches and immersed in 2.5ml of 2% glutaraldehyde for 4 hours to inactivate and fix the spores. The fixative was removed and the swatch was immersed in 5 ml of phosphate buffer for 10 minutes. The fixed swatches were dried using an ethanol series of 50%, 70%, 90% and 100% for 10 minutes at each concentration, then left for 24 hours to air-dry completely. Individual swatches were mounted on 2.5cm aluminium stubs and sputter coated with gold (Edwards Sputter Coater, S150B). The stubs were viewed using a SEM (Carl Zeiss Evo HD 15), in high vacuum with a beam accelerating voltage of 5-7 Kv for magnifications between 2500 and 50, 000.

3.3.6 Assessment of the healthcare laundry policy using experimental (100% cotton) sheets naturally contaminated with *C. difficile* spores

3.3.6.1 Ethical Approval

Ethical review was carried out internally, by the De Montfort University Health and Life Sciences' Research Ethics Committee (Reference: 714). NHS ethical review was also sought and a favourable opinion obtained from 'Nottingham 2' NHS Research Ethics Committee (Reference: 11/EM/0002). Local Research and Development permission was obtained from the trust responsible for the NHS hospital research site (Reference: UHL10003). The participating hospital allowed access to a *C. difficile* isolation ward with a letter of permission and a meeting with the ward manager.

3.3.6.2 Media

Braziers CCEY selective agar (Lab160, LabM, UK) was used to ensure only *C. difficile* spores were isolated from the samples. The Brazier's agar contained the selective antibiotic Cycloserine and Cefotoxin supplement (X093, LabM, UK) and egg yolk emulsion (X073, LabM, UK). The Brazier's agar was stored at 4°C and used within one week.

3.3.6.3 Storage and collection of the experimental (100% cotton) sheets

The storage of blue experimental (100% cotton) sheets after use in the ward, was identical to that of standard infected linen, to ensure a realistic quantification of *C. difficile* spore burden before washing. A set of six experimental sheets were delivered weekly to the ward. The sheets were used on CDI patient beds, changed daily and when soiling had occurred. Incontinence pads were in use where necessary. After use, the sheets were placed in alginate bags and sealed using a plastic tie, provided with each alginate bag. The sealed alginate bags were put into standard red 'infected-linen' labelled plastic non-permeable bags and closed by a single knot. The sealed bags were placed into a labelled box within the used linen room for the ward. The sheets were collected once every 24 hours, in accordance with standard practice at the site and transferred to a secure outside waste compound. They remained there overnight in a box adjacent to standard infected linen and were collected the following morning for sampling.

3.3.6.4 Sampling

All linen bags were opened within a class two cabinet (Class II, Lab Guard, UK) and each sheet with visible soiling was numbered using a permanent marker. Soiled regions were identified, from which three 25cm² sections could be sampled, leaving adjacent 25cm² soiled regions for sampling post-wash. The sample swatches were placed into 50ml falcon tubes. The sheet was placed

back into the alginate bag, resealed with the tie and put into the red 'Infected Linen' bags. The bags were then transported to the laundry the same day.

The sample swatch had 30mls of MRD added to the tube and viable spores were recovered by the vortex method (section 3.2.1 B). After 5 minutes the swatch was removed and the suspension was heat shocked in a water bath at 80°C for 10 minutes. The resulting 30ml suspensions were vacuum filtered as previously described (section 3.2.4). Agar plates were then incubated anaerobically at 37°C for 48 hours. If there were no colonies, the plates were left for an additional 48 hours.

The sampled sheets were transported to the laundering facilities immediately after sampling and washed with other infected linen according to the HSG (95) 18 Hospital arrangements for the laundering of infected linens (HTM 01-04).

Post-wash samples were processed by the same method for pre-wash samples, however, the spore suspensions obtained were not heat shocked. The spores which had survived the washing process may have become more susceptible to damage from heating and there was less likelihood of isolating other organisms. Furthermore, post-wash samples suspensions were combined so that the three 30ml suspensions generated from each sheet were filtered together, as recommended by the collaborating laboratory. The sample size was

calculated by Dr Taub of the National Institute of Health Research, who advised 36 *C. difficile* spore contaminated sheets were needed; if all were positive before laundering and negative after laundering, the sample size would provide sufficient evidence to state that the laundry process was effective.

3.3.6.5 Industrial Laundering

At the collaborating commercial laundry, the bags were transferred to the 'dirty' side of the facility and the unopened alginate bags were loaded into a WE for a wash cycle at 75°C for ≥3 minutes with 8 minutes mixing time, with the industrial detergent system used in the simulated washes (section 3.2.4). The sheets were then transported to the ironer bed where they were pressed and dried at 175°C with 4 bars of pressure for three seconds (calendaring). The laundered experimental sheets were then collected the following day for repeat sampling, and the second of each paired region was cut and sampled from each sheet as previously described (section 3.2.5.4).

3.3.6.6 Identifying clinical strains

All presumptive *C. difficile* positive plates were confirmed by the Gold standard latex agglutination and long-wave UV fluorescence test. All confirmed *C. difficile* plates were then sub-cultured and stored on cryobeads at -20°C and -80°C for further investigations. Ribotyping was performed by PCR with capillary gel electrophoresis (CE-Ribotyping), on all positive samples at the Public Health England (PHE) laboratory in Newcastle. CE-Ribotyping has been shown to

reduce inter-laboratory variability (Fawley *et al.*, 2015). In brief, the 16S-23S intergenic spacer region was amplified by PCR, with the resulting fluorescent tagged fragments analysed by passing through a submillimetre gel capillary using an electric current. With the ends of the capillary within an aqueous buffer the fragments are run with a control sample for size comparison and size data is collected. The size of the intergenic spacer regions can then be compared to a library of known isolates.

3.3.7 Healthcare laundry survey for care facilities

3.3.7.1 Overview and ethical approval

The survey questions (Appendix II) were designed to get an overview of how care facilities met the minimum requirements of the HTM 01-04 social care volume of the laundry policy. In addition, an assessment of whether the facility met EQR was made, by considering cycle temperature, machine/detergent type, sluice or pre-wash cycles, annual verification and the presence of infectious patients. Ethical review was carried out internally as an amendment to an approved study by the De Montfort University Health and Life Sciences' Research Ethics Committee, a favourable opinion was obtained (Reference: 714).

3.3.7.2 Selection method

The care facilities were chosen from an online database, 'www.Care.com', using the following procedure: 20 English counties were randomly picked by computer, 10 participants were identified on the website per county that were owned or managed by different providers and had a range of resident numbers and review scores. A range of 1-3 facilities per page of listings were selected, until 10 had been identified per county, with 200 facilities in total. The introductory letter was addressed to the manager or responsible person for each site.

3.3.8 Statistical analyses

All statistical analyses were performed with IBM SPSS v. 22. The significance value for all tests was set at $p \leq 0.05$. The Shapiro-Wilk test for the normality of the distribution and Levene's test for equality of variance were used to determine whether data could be analysed by independent one-way analysis of variance (ANOVA) or independent t-test. Where data were assessed by ANOVA, Tukey's post-hoc tests were performed to make pair wise comparisons. Where the assumption of a normal distribution of the data was violated, the non-parametric Mann-Whitney *U* test or Kruskal-Wallis (with *post-hoc* multiple comparisons and adjusted significance) were performed. Each investigation was repeated in triplicate on two separate occasions, unless otherwise stated. The survey data were collated in Microsoft Excel (2013) to ascertain frequencies and

a Spearman Rank was performed to analyse the association between wash temperature and the number of different types of infection. In addition, an independent t-test was performed to assess whether the maximum wash temperature was different at sites with and without CDI.

3.4 Results

3.4.1 Recovering *C. difficile* spores from air-dried cotton swatches

The highest level of recovered spores was $3.33 \log_{(10)} \text{ cfu}/25\text{cm}^2$, of the original $\sim 4 \log_{(10)} \text{ cfu}/\text{ml}$ inoculum, which was achieved by the vortexing method (Figure 3.1). However, the vortexing method was not statistically significantly better at recovering spores than stomaching on high for 1 minute ($3.08 \log_{(10)} \text{ cfu}/\text{ml}$, $p > 0.05$) or the hand shake method ($3.08 \log_{(10)} \text{ cfu}/\text{ml}$, $p > 0.05$). The original inoculum was $4 \log \text{ cfu}/\text{ml}$ per swatch. All other methods recovered significantly fewer spores ($< 3 \log_{(10)} \text{ cfu}/\text{ml}$ spores, $\chi^2 = 67.317$, $p \leq 0.05$).

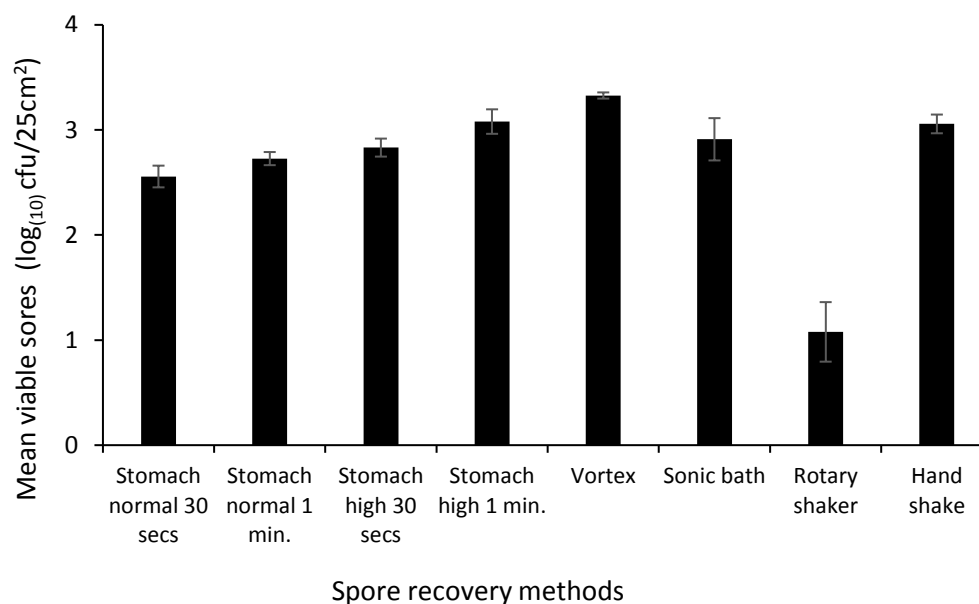


Figure 3.1. *C. difficile* spores recovered from air dried cotton swatches (mean \pm SE, $n=4$).

The stomaching method showed a trend of increased spore recovery rates with increasing time and speed (Figure 3.1). However, this was only statistically significant when comparing the stomaching on high for 1 minute with stomaching on normal for 30 seconds ($p \leq 0.05$).

3.4.2 Soiling effect on *C. difficile* spore recovery from swatches

The highest level of recovered spores was obtained using the Vortexing method, with high and low soiling having 4.48 \log_{10} cfu/ml and 4.41 \log_{10} cfu/ml recovered respectively from the original inoculum of 5 \log_{10} cfu/ml spores (Figure 3.2). Vortexing recovered a greater proportion of the spores when soiling was present, compared to vortexing without the addition of soiling

(section 3.3.1), with vortexing at no soil, low soil and high soil recovering 20%, 26% and 31% of spores, respectively.

There were significantly more spores recovered by the vortexing method, than by the stomaching method, with low soiling and with high soiling ($\chi^2=29.33$, $p\leq 0.05$). Using the vortexing method to compare recovery of spores from the white NHS (100% cotton) sheet swatches and blue experimental (100% cotton) sheet swatches, there was a statistically significant difference of 0.29 $\log_{(10)}$ cfu/25cm² at both low and high soiling ($\chi^2= 26.593$, $p\leq 0.05$, $n=6$).

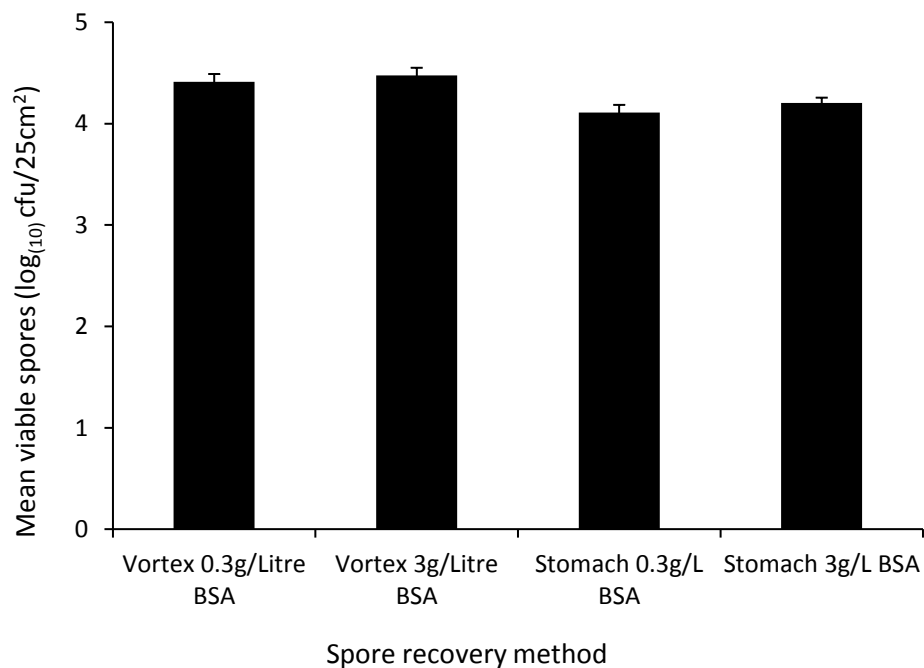


Figure 3.2. *C. difficile* spores recovered from NHS (100% cotton) sheet swatches with BSA soiling at low (0.3g/L) and high (3g/L) (mean \pm SE, $n=6$).

3.4.3 Quantification of the survival of *C. difficile* spores in simulated washes

All data included in statistical analyses were from validated cycles, where the data logger recorded the required time and temperature (71°C for ≥ 3 minutes + 8 minutes mixing time). The time the wash remained at 71°C was 13 minutes ± 30 seconds. In the control cycle (without detergent), there were significantly fewer spores recovered, compared to the original inoculum ($7 \log_{(10)} \text{cfu}/25\text{cm}^2$), for *C. difficile* NCTC 11209 ($4.95 \log_{(10)} \text{cfu}/25\text{cm}^2$, $U=26.39$, $p \leq 0.05$) and ribotype 001/072 ($5.27 \log_{(10)} \text{cfu}/25\text{cm}^2$, $t=19.39$, $p \leq 0.05$). In addition, the number of recovered spores after the control cycle were not significantly different when comparing NCTC 11209 and ribotype 001/072 ($p > 0.05$). Without detergent, the effects of temperature and agitation alone were unable to reduce the number of spores by $> 2 \log_{(10)} \text{cfu}/25\text{cm}^2$, leaving the swatches heavily contaminated with $4.95 \log_{(10)} \text{cfu}/25\text{cm}^2$ *C. difficile* spores (Figure 3.3).

In contrast, the number of spores that could be recovered from swatches after the detergent cycles, were markedly reduced. The resulting $\text{cfu}/25\text{cm}^2$ recovered was significantly lower than the original inoculum ($7 \log_{(10)} \text{cfu}/25\text{cm}^2$) for both NCTC 11209 ($0-4 \text{cfu}/25\text{cm}^2$, $p \leq 0.05$) and ribotype 001/072 ($0-9 \text{cfu}/25\text{cm}^2$, $p \leq 0.05$). The use of detergent also resulted in a significantly lower number of spores recovered, than the control cycles for NCTC 11209 ($p \leq 0.05$) and ribotype 001/072 ($p \leq 0.05$). Again, the number of spores recovered

after the detergent cycle was not significantly different between the two strains (NCTC 11209 0-4 cfu, ribotype 001/072 0-9 cfu, $p>0.05$).

In regard to the level of cross-contamination, there were similar numbers of spores recovered from previously sterile swatches in the control cycles for both NCTC 11209 and ribotype 001/072 (2.72 vs 2.89 $\log_{(10)}$ cfu/25cm², $p>0.05$). Significantly lower levels of cross-contamination were found in cycles which included detergent, for both NCTC 11209 and ribotype 001/072 (0.09 $\log_{(10)}$ vs 0.16 $\log_{(10)}$ cfu/25cm², $p\leq 0.05$), with a range of 0-8 and 0-14 cfu/25cm², respectively (Figure 3.3).

In addition to the validated industrial cycle data, the swatches from a single industrial cycle which did not reach 75°C for the minimum time were recovered. This cycle showed an increased number of spores recovered in a detergent cycle, when 75°C was maintained for only 4 minutes instead of 13 minutes (NCTC 11209, 1.84 $\log_{(10)}$ cfu/25cm², range 62-100 spores). There were no viable spores found inside the drum of the washing machine, between washes or after the terminal decontamination protocol (section 3.2.4).

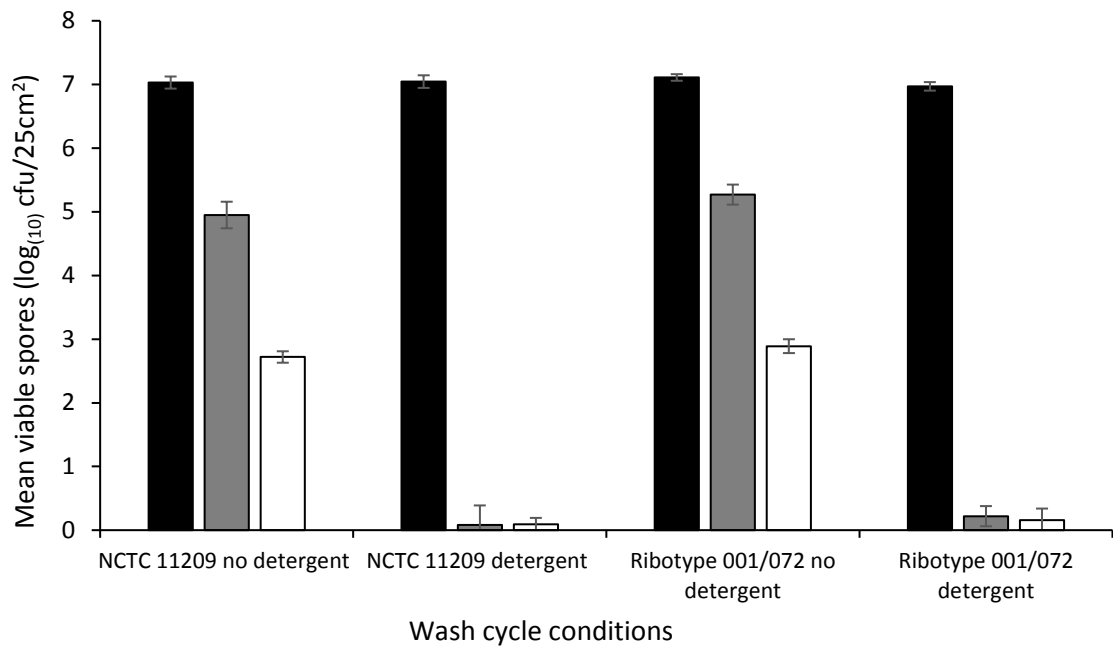


Figure 3.3. *C. difficile* spores recovered: the original inoculum (\log_{10} cfu/ml) (■), per inoculated swatch (■) and per previously sterile swatch (□). Strain types NCTC 11209 and ribotype 001/072 (mean \pm SE, $n=4$).

3.4.4 SEM of NHS (100% cotton) sheet swatches

The images of old and new swatches cut from white NHS (100% cotton) sheets showed that the new NHS (100% cotton) sheet material had most of the fibres running parallel, with no evidence of frayed or ragged strands (Figure 3.4 A).

The woven fibres looked relatively smooth, intact and large. In contrast, the old NHS (100% cotton) sheet material, which was representative of a standard sheet in use within the rental linen system, had a rougher aspect, with ragged filaments standing up from the weave and the fibres looked smaller (Figure 3.4 C).

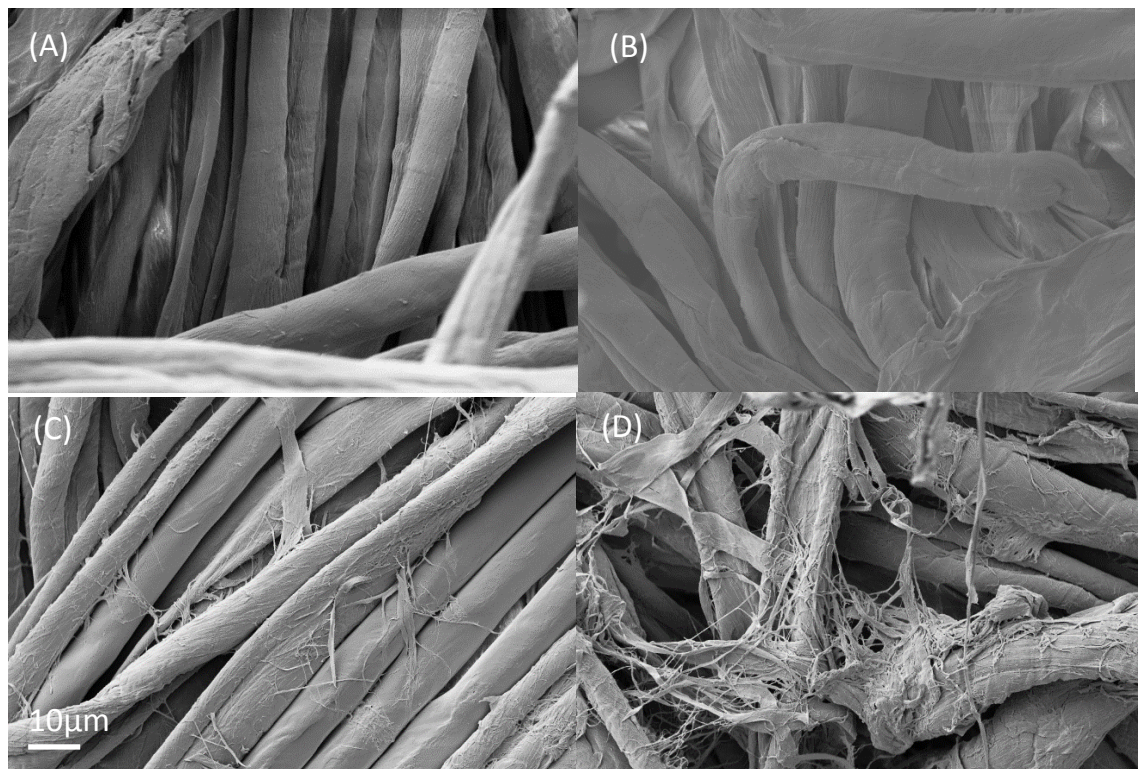


Figure 3.4. Swatch samples (1cm^2) from NHS bed sheets at 2500 magnification (A) new clean swatch, (B) new heavily soiled swatch (0.1ml of 3g/l BSA/ 25cm^2), (C) old clean swatch, (D) old heavily soiled swatch (0.1ml of 3g/l BSA/ 25cm^2).

A swatch from a non-validated simulation of a healthcare wash cycle where the temperature was not maintained for the full 13 minutes was viewed (Figure 3.5). There were many spores in large clumps, perhaps within the soiling with which they were inoculated (Figure 3.5). There was a single spore which looked as though it had been lysed either by the detergent or thermal disinfection or a combination of both (Figure 3.5 C).

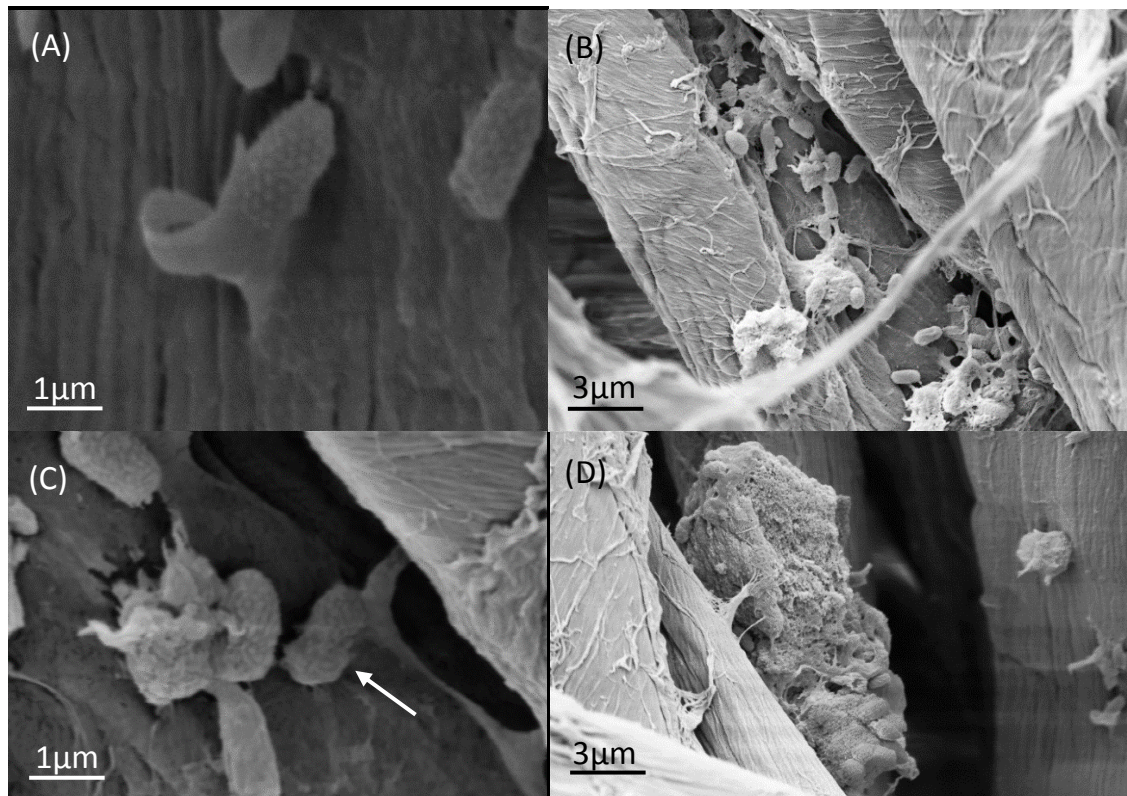


Figure 3.5. *C. difficile* spores on a NHS (100% cotton) sheet swatch inoculated with 0.1ml of $8 \log_{(10)}$ cfu/ml test suspensions containing 3g/L BSA. Swatch air dried for 24 hours, then washed in a failed simulated healthcare wash at 71°C with industrial detergent (held at 71°C for 5 minutes instead of 13 minutes). (A) spore, (B) many spores left on the swatch after washing, (C) further magnification area in (B) with a possible damaged spore (white arrow), (D) large clump of spores (soiling or co-aggregation).

3.4.5 Assessment of the healthcare laundry policy using experimental (100% cotton) sheets naturally contaminated with *C. difficile* spores

The swatches removed from the naturally contaminated sheets ($n=3$) had an average of 51 cfu/25cm² ($1.7 \log_{(10)}$ cfu/25cm²), with a range of 2-158 cfu/25cm² (Table 3.2). The post-wash swatches (washed at $\geq 71^\circ\text{C}$ for 3 minutes + 8 minutes mixing, dried and finished) still had an average of 33 cfu/25cm² ($1.25 \log_{(10)}$

cfu/25cm²). The post-wash average was calculated from a combined 90ml sample for each sheet (>100 cfu/75cm²). This equated to a post-wash reduction of 18 cfu/25cm² (or 0.45 log₍₁₀₎ cfu/25cm²), or 40% reduction in spore load after washing. Subsequent ribotyping of the isolates recovered before and after washing, showed they were indistinguishable from each other and had only a single minor difference from ribotype 001/072 (PHE, Newcastle). This suggests that the spores recovered post-wash, were present before the wash and were not a result of contamination during the wash cycle.

Table 3.2. *C. difficile* spores recovered from swatches (25cm²) of blue experimental (100% cotton) sheets, before and after washing to NHS healthcare policy minimum standards HTM 01-04 (*n*=3).

Sample	Mean spore count	Range	Standard error	Ribotype
Pre-wash	51 cfu/25cm ²	2-158	33.23	001/072
Post-wash	33 cfu/25cm ² (100 cfu/75cm ²)	-*	-*	001/072

* = cfu/75cm² recorded at maximum count for each sheet (>100).

3.4.6 Healthcare laundry survey for care facilities

There were 30/200 (15%) completed questionnaires returned from the healthcare facilities, covering 18 counties across England, with a maximum of 3 surveys for a single county. There were only seven (23.3%) facilities that were not currently caring for people with an infectious disease, whereas more than half of respondents were dealing with three or more types of infectious disease (17/30, 56.7%). The infectious diseases included *C. difficile* 17/30 (56.7%), MRSA 22/30 (73.3%), fungal infections 21/30 (70%) and other infectious disease - skin condition 1/30 (3.3%). All facilities which were dealing with *C. difficile* were also dealing with at least one other type of infectious disease.

The bed sheet material used at each facility was either cotton 15/30 (50%), poly-cotton blend 13/30 (43.3%), or both types 2/30 (6.67%). Use an onsite laundry was by far the most common method to launder bed sheets (26/30, 86.7%), with only four choosing a commercial laundry provider. The majority (75%) were on a linen rental contract whereas one facility had their own linen laundered and returned.

Of those using an onsite laundry, 20/26 (76.9%) used an industrial grade washing machine, 1/26 (3.8%) used a domestic grade washing machine, with 5/26 (19.2%) facilities not providing data. The facility using a domestic machine also used domestic detergent, Fairy non- bio (Proctor and Gamble), but did not

provide data on any infectious diseases present at the facility. Considering laundry detergent, there were 18 different brands used over the 26 sites, with a maximum frequency of two sites using the same detergent brand. The limited information provided suggests two were using domestic grade detergents, 21/26 (80.7%) were using industrial grade detergent and three did not provide any information on the type.

The maximum temperature of the wash cycle was 90°C, while the minimum was 45°C (Table 3.3). There was no statistically significant association between the temperature of the wash, compared with the number of types of infectious disease within the facility ($r=-0.222$, $p>0.05$). There was a trend toward a higher maximum temperature in those facilities without CDI present, but this was not statistically significant ($t=0.744$, $p>0.05$).

Regarding wash cycle length, 4/26 (15.4%) had a cycle length under 1 hour, the other respondents were split evenly with 11/26 (42.3%) having a cycle between 1 hour and 1 hour 30 mins and 11/26 (42.3%) having a cycle between 1 hour 30 mins and two hours. None of the respondents provided information about the length in minutes of the main thermal disinfection cycle, i.e. maintained for ≥ 3 minutes at $\geq 71^\circ\text{C}$ or an alternative disinfection option (ozone or wash additives etc.)

Table 3.3. Maximum temperature of the main wash cycle and the frequency of sites with CDIs, that wash infected linen at each temperature.

Temperature range (°C)	Frequency temperature used within healthcare facilities	Frequency of CDI
40-49	3/26 (11.5%)	2/3 (66%)
50-59	1/26 (3.8%)	0/1
60-69	8/26 (30.8%)	5/8 (62.5%)
70-79	4/26 (15.4%)	2/4 (50 %)
80-89	1/26 (3.8%)	0/1
90-99	7/26 (26.9%)	3/7 (42.9%)
Unknown temperature	1/26 (3.8%)	1/1

The information regarding other parts of the cycle and safety measures was provided in all cases. Over half of the facilities were using a sluice cycle (16/26, 61.5%), the majority were using alginate bags (19/26, 73.1%), which are required for infected linen, unless loading is automated, and eight performed a pre-wash cycle (30.8%). However, only five facilities used alginate bags, sluice cycles and pre-wash cycles for infected linen (19.2%). Tumble drying was the drying method of choice for all but one of the facilities (25/26, 96.2%). Just over half of the facilities confirmed that they had an annual verification of the washing system (15/26, 57.7%), whereas 2/26 (7.7%) did not have an annual

verification. Information regarding annual verification was not provided for nine facilities (34.6%). Of the 20 facilities using industrial machines, only 12 said they were also using industrial detergent and getting an annual validation of the disinfection cycle. Annual validation of the disinfection part of the cycle, whether thermal or chemo-thermal, has been a requirement of CFPP01-04 and is still required by HTM 01-04.

3.5 Discussion

3.5.1 Recovering *C. difficile* spores from air dried cotton swatches with and without soiling

The agitation method used to recover spores from NHS (100% cotton) sheet swatches significantly affected the number of *C. difficile* spores recovered (Figure 3.1). The recovery rates from each of the methods varied widely from 1.08 log₍₁₀₎ cfu/25cm² for rotary shaking, to 3.33 log₍₁₀₎ cfu/25cm² for vortexing. Without the addition of simulated soiling there was no significant difference between vortexing, hand shaking and stomaching on high for 1 minute ($p>0.05$). There was a trend within the stomaching method for the rate of recoverable spores to increase with increasing time and increasing speed of agitation. This was only statistically significant when time and speed were both increased ($p\leq 0.05$). The rotary shaker recovered a significantly lower number of spores

than all other methods, which contrasted with Jenkins and Sherbourne (2005) who found that rotary shaking (10 minutes) was particularly efficient when used with glass rod agitation (20 seconds) recovering 60%-90% of inoculated bacterial cells. Interestingly, none of the methods assessed in section 3.2.2., were able to recover greater than 31% of the spores inoculated. It must be considered that the spores were still adhered to the fabric after the recovery had been performed, as there were no measures employed which could have inactivated the spores. This conclusion was also reached by Rose *et al.* (2004) where fewer *B. anthracis* spores were recovered from cotton swabs which had been used to sample air-dried stainless-steel coupons, compared to direct inoculation of the swab with immediate recovery. The air drying and extended time between inoculation and swabbing, was likely to have promoted adherence to the coupon, as there were no methods employed to inactivate the spores. However, there were no further samples taken from the coupon to assess the number of viable spores remaining.

Joshi *et al.* (2012) also demonstrated a variable ability for *C. difficile* spore strains to adhere to surfaces such as stainless-steel. The authors report a large range in terms of proportion of spores adhering to stainless-steel, with 14%-80% of spores not recovered (conversely 20%-86% recovered) after pressing an inoculated steel disk onto 16 BHI agar plates in succession. The exact factors which mediate spore adherence to surfaces are currently

undefined, but there is growing evidence which suggests spore surface components, such as the exosporium, play a role in attachment to organic and inorganic surfaces. It has been shown that when the exosporium has been removed, there is a reduction in adherence to human epithelial cells and reduced hydrophobicity (Paredes-Sabja and Sarker, 2012). It could be hypothesised that there are components of the spore surface which are directly adhering to the cotton fibres of the swatches, preventing the recovery of the greater proportion of spores.

Conversely, fibre binding studies have shown MSSA and MRSA vegetative cells have a relatively low level of binding to cotton fibres, 2% and 1% respectively (Takashima *et al.*, 2004). In comparison, 5% of *P. aeruginosa* vegetative cells were found to bind to cotton fibres. The method employed in recovering the cells was different to the present study, as it was a quantification of the cells left in broth media after agitation in the presence of cotton fibres. The authors were unable to assess woven cloth by their method, as the broth was absorbed into woven cloth and cell counts were artificially increased. There was also no comparison of the effect of soiling on the recovery of bacterial cells.

In the present study soiling was introduced into the comparison of spore recovery methods, to simulate more realistic conditions within the laundry; consequently, vortexing recovered significantly more spores than stomaching

and hand shaking at low and high soiling ($p \leq 0.05$) (Figure 3.2). It is not clear why the result should be different to the previous comparison, unless the presence of soiling affected the recovery. The objective of the comparison was to choose an efficient method of recovering spores in presumably heavy soiling conditions, so vortexing was the method chosen for use in the simulation of a healthcare standard wash (3.2.3.) and for the assessment of the healthcare laundry policy using naturally contaminated linens (3.2.4). Vortexing has also been used in other studies assessing the efficacy of the minimum laundry conditions required by HTM (95) 18, for example Orr *et al.* (2002) assessed laundry efficacy to decontaminate enterococci, as did Fijan *et al.* (2007) and Fijan *et al.* (2010) which both assessed the number of recoverable bacterial cells after low temperature washes, however, the time and speed of vortexing was not reported. Rose *et al.* (2004) directly inoculated *B. anthracis* spores onto cotton swabs and immediately recovered the spores by vortexing on 'high' for 2 minutes. In contrast to the present study, an average 93.9% of the *B. anthracis* spores were recovered and soiling was not considered. The reasons for the difference in proportions could be the increased duration of vortexing (2 minutes vs 1 minute), differences between species of spore-former or immediate sampling with no air-drying after inoculation. However, the present study agrees with Rose *et al.* (2004) when comparing vortexing with sonication for recovering spores from cotton swabs, after sampling air-dried inoculated

steel coupons. Vortexing for 2 minutes recovered a significantly higher proportion of the spores than sonication for 12 minutes (41.7% vs 13.6%) in comparison to vortexing for 1 minute and sonication for 1 minute (31% vs 8.2%).

It is of note, that the current healthcare laundry policy, HTM 01-04, suggests the use of 'agitation' to recover *B. cereus* spores for annual seasonal microbiological assessments (Department of Health, 2016a). Stomaching for 1 minute was recommended by the laboratory which was responsible for microbiological assessment of the commercial laundry investigated in this study (section 3.2.5). Therefore, the proportion of viable spores (*B. cereus* or *C. difficile*) that any laboratory may be able to recover, may vary dependent on the type and duration of agitation method chosen.

3.5.2 Quantification of the survival of *C. difficile* spores in simulated washes

The wash cycles used in this investigation all reached 75°C, and were held at ≥71°C for >3 minutes (+ 8 minutes mixing time) as required by thermal disinfection conditions of the policy in place at the time (CFPP 01-04) and the updated HTM 01-04. It was demonstrated that the use of detergent was an important factor in significantly reducing the numbers of *C. difficile* spores, this was true for both strains assessed (Figure 3.3). There was a >6 log₍₁₀₎ reduction

in the number of viable spores recovered from swatches washed using the industrial detergent system in the simulated HTM 01-04 compliant healthcare cycle. The $>6 \log_{(10)}$ reduction still remained true when it was considered that the spore recovery method was able to recover an average of 31% of the viable spores remaining on the swatch.

In the control cycle without the industrial detergent system, the thermal disinfection and agitation were found to be inadequate to affect a $>5 \log_{(10)}$ reduction in *C. difficile* spores. There was still a significant number of spores recovered from the swatches (Figure 3.3). This demonstrates that in the event there is a failure to include detergent during a healthcare cycle, there may be a significant viable spore burden on processed linen.

Cross-contamination was seen in both the control wash and detergent wash, where spores were being removed from the inoculated swatches and then transferred to other material present in the drum (Figure 3.3). Cross-contamination was significantly higher when the detergent system was not included in the cycle.

Chemo-thermal disinfection proved to significantly better at decontaminating the swatches, but even in an optimized wash there were still $0.1-1 \log_{(10)}$ cfu/25cm² which would fail the microbiological validation requirement: no pathogenic bacteria. This suggests that the policy could provide

better guidance, particularly when it comes to detergent usage and new or emerging disinfection technologies.

The healthcare wash cycle simulations provide quantification and further insight to the understanding of whether *C. difficile* spores survive the thermal and chemo-thermal conditions which can be applied by laundries (whether commercial, or onsite in a care facility). The findings of the simulation verify the work by Hellickson and Owens (2007), who demonstrated the unquantified presence of *C. difficile* spores on inoculated and previously sterile swatches washed in a simulated healthcare wash cycle. The present study goes further, in establishing that in the simulation conditions described, the use of detergent significantly reduces the number of viable spores recovered by 6-7 $\log_{(10)}$ on inoculated swatches and reduces cross-contamination by >2.5 $\log_{(10)}$ on previously sterile swatches. The 0.1 – 1 $\log_{(10)}$ cfu/25cm² that may remain on swatches after a simulated healthcare wash cycle was shown to be a conservative estimate, due to the inability to recover 100% of viable spores present with the method used. There may be a proportion (~69%) of spores still adhering to the swatch (Figure 3.2).

Despite the findings being a conservative estimate, there was agreement with work by Lakdawalla *et al.* (2011), who demonstrated that *C. difficile* 1-3 $\log_{(10)}$ cfu/100cm² spores were recoverable after a CTW healthcare cycle which met the minimum thermal disinfection conditions of HTM 01-04. Unfortunately,

Lakdawalla *et al.* (2011) did not quantify the original spore load and could not calculate the reduction in the number of *C. difficile* spores present after washing.

In work by Alfa *et al.* (2013) on bed pan washers, it was demonstrated that an 85°C (1 minute) thermal disinfection cycle was unable to completely decontaminate bed pans inoculated with ~5-6 log₍₁₀₎ cfu/site *C. difficile* spores. The authors concluded that it was the combined effects of the detergent used, mechanical agitation and thermal inactivation which finally gave a significant reduction of *C. difficile* spores. In brief, the authors put vials of *C. difficile* spores through a bed pan wash cycle, alongside test bed pans. After thermal disinfection at 85°C for 1 minute, there was a reduction of 1.46 log₍₁₀₎ cfu/ml, leaving 5.32 log₍₁₀₎ cfu/ml viable spores within the vials. In addition, inoculated bedpans demonstrated a reduction in *C. difficile* spores of 4.45 log₍₁₀₎ cfu/site leaving an average of 1.15 log₍₁₀₎ cfu/site. With the addition of an alkaline detergent, in combination with the thermal disinfection, there were no viable spores recovered. The combined effects of detergent, agitation and thermal disinfection agreed with the present study, whereby the addition of detergent significantly reduced the number of viable spores recovered compared to relying on thermal disinfection and agitation alone (Figure 3.3). However, the bed pans inoculated by Alfa *et al.* (2013), were a smooth inorganic plastic material and may have different adherence properties to organic cotton fibre

swatches. The bed pan wash tests were followed up by Macdonald *et al.* (2016), where it was again demonstrated that thermal disinfection alone, within a bed pan washer, was not sufficient to decontaminate $\sim 6 \log_{(10)}$ *C. difficile* spores within a vial placed into a standard wash cycle which reached 92°C and the total cycle time was 11-18 minutes.

More recently, Mackay *et al.* (2017) compared washing ambulance uniform swatches, artificially contaminated *C. difficile* spores, at 30°C and 60°C in a Miele PW6055 (commercial) machine with Cleanline biological washing powder. Reusable mop head strands were also inoculated and washed at 30°C and 95°C. Soiling was simulated with defibrinated horse blood. Cells and spores were recovered by stomaching on high for four minutes. Viable *C. difficile* spores were recovered from all swatches washed at 30°C and 60°C, where there was a 0.3 $\log_{(10)}$ increase of viable spores recovered at 30°C in unsoiled washes and 1.1 $\log_{(10)}$ reduction in soiled washes, leaving 6.73 $\log_{(10)}$ cfu/swatch and 6.20 $\log_{(10)}$ cfu/swatch, respectively. Furthermore, at 60°C there was 1.6 $\log_{(10)}$ (unsoiled) and 1.9 $\log_{(10)}$ (soiled) reduction of viable spores leaving 5.23 $\log_{(10)}$ (unsoiled) and 5.27 $\log_{(10)}$ cfu/swatch (soiled).

Agreeing with the present investigation and others previously described (section 3.1.2) there was cross-contamination of *C. difficile* spores to previously sterile swatches added to the wash, at the limit of detection, of around 3.398 $\log_{(10)}$ cfu/ml at both 30°C and 60°C. *C. difficile* spores were recovered from all 6

mop strands washed at 30°C (between 1.1 – 2.2 log₍₁₀₎ reduction) and one of the three unsoiled mop strands washed at 95°C, with a 2.36 log₍₁₀₎ reduction leaving 4.50 log₍₁₀₎ viable spores on a mop strand washed at 95°C in an industrial machine. The study was limited by the high limit of detection, the Miles and Misra technique was unable to detect spores at <3.4 log₍₁₀₎ cfu/ml. The other mop strands tested, may still have had viable spores at levels below the detection limit, but which could go on to contaminate the environment during use.

Whether these low number of spores could go on to contribute to the environmental contamination and produce active CDI or asymptomatic carriage in a patient, is beyond the scope of this work. However, this could be further examined by inoculating NHS (100% cotton) bed sheets at an appropriate concentration (i.e. 1-2 log₍₁₀₎/25 cm²) and conducting bed-making in an experimental hospital room. Samples from the environment could be taken from surfaces at various distances and air samples; this would assess how sheets contaminated with low levels of spores may contribute to the contamination of the near-patient environment. As previously discussed environmental contamination is linked to CDI rates, and bed-making is highly likely to be performed after terminal or general cleaning has been undertaken.

In summary, the comparison of cycles with and without detergent demonstrates the importance of the detergent as a key method of

decontaminating linen, which had been inoculated with *C. difficile* spores, when processed in a simulated healthcare wash cycle. Several possible mechanisms were at work, which must be considered. The surfactant within the detergent may be aiding removal of spores into the wash water during agitation, there may be sporicidal activity on the part of the detergent or the additives (sodium hypochlorite bleach and peracetic acid). The thermal disinfection cycle may work synergistically with sporicidal agents within the wash cycle and have an accumulative effect. It would be beneficial to further explore the interactions between temperature, chemical agents and the rinsing and removal of spores from inoculated swatches to understand how the different factors aid in decontamination. The relationship between the thermotolerance of spores, sporicidal activity of detergents and the effects of soiling were explored further (section 4.2). The findings may be useful for developing a more effective and evidence-based disinfection protocol for healthcare linen, particularly with the drive to reduce energy use and water consumption (Textile Services Association, 2015). Changes to the temperature, time or water levels need to be compensated for with increases in the other key components of an effective wash cycle e.g. sporicidal chemicals, duration of agitation (Bockmühl, 2017) .

The optimised programme, along with the industrial detergent system used in this healthcare wash simulation were successful at significantly reducing *C. difficile* spores from inoculated swatches. However, there were still low levels

of spores recovered from inoculated swatches and previously sterile swatches, which may contribute to environmental contamination in a healthcare setting. It is also worth noting that the entire wash protocol was performed under controlled conditions, *in situ*, there may be time pressures, varying levels of soiling, over-loading of the drum, or other human-related errors which may reduce the effectiveness of a healthcare wash.

3.5.3 SEM of NHS (100% cotton) sheet swatches

SEM images of new and old NHS (100% cotton) swatches, provided a qualitative assessment of the roughness and where soiling may be seen when present. As expected the new NHS (100% cotton) swatches appeared less rough and the fibres were more intact i.e. bigger and smooth (Figure 3.4). In contrast, the old NHS (100% cotton) swatches were smaller, with thin fibres running between the large main fibres. This could be evidence of the wear and tear the sheets are subjected to during repeated use and regular high-temperature washing. The old NHS (100% cotton) swatch which was heavily soiled, had thin fibre filaments forming a web across and between the larger fibres, this may have been due in part to the soiling added to this swatch; however, the new NHS (100% cotton) sheet swatch with soiling, exhibits none of the filaments. This suggests the thin filaments linking fibres into a web may be due to cotton fibres, rather than soiling. As roughness can be a factor in removal of spores from surfaces,

particularly in the presence of soiling, the roughness could be further assessed by the use of a roughness measure test (Gonzalez *et al.*, 2017).

3.5.4 Assessment of the healthcare laundry policy using experimental (100% cotton) sheets naturally contaminated with *C. difficile* spores

This study provides the first estimate of the level of *C. difficile* spore contamination on a naturally contaminated sheet and was surprisingly low considering the published estimates of spore-shedding in CDI patients: 5-7 \log_{10} cfu/g faeces (Riggs *et al.*, 2007). It was demonstrated that the levels of *C. difficile* spores present on the sheets before washing (51 cfu/25cm²), were comparable to those found after washing (33 cfu/25cm²); there were not enough sheets returned to assess the data statistically (Table 3.2). The thermal disinfection parameters performed in a WE described by the HSG (95) 18 policy and continued through to HTM 01-04, were unable to significantly reduce the natural contamination level of *C. difficile* on the sheets tested.

Furthermore, the industrial detergent system used at the collaborating laundry was the same system assessed during the simulations (section 3.2.4). During the simulation the detergent system was able to significantly reduce spore contamination, compared to the original inoculum and compared to the control cycle without detergent (Figure 3.3). When used *in situ*, the detergent

was unable to affect a significant reduction in spores. Furthermore, the sheets were exposed to additional repeated high temperature cycles, through drying and calendaring, beyond what was possible in the simulations. The repeated heating should have aided decontamination (Rodriguez-Palacios and LeJeune, 2011; Wilson *et al.*, 2007).

At the collaborating commercial laundry, the WE cycle (without drying or calendaring) is equivalent to the pre-wash conducted before infected linen is added to a CTW for a main wash. The WE pre-wash is specifically conducted to decontaminate infected laundry before it comes in to contact with other textile items. The regular infected linen loads are processed by WE (71°C for ≥3 minutes + 8 minutes) as a pre-wash, followed by a main wash cycle in a CTW (71°C for ≥3 minutes + 8 minutes) at which time further reductions in spores may have occurred.

The experimental sheets (100% cotton) used in this study were washed in a WE cycle for a high temperature wash cycle (~90°C), where the minimum thermal disinfection conditions were met, and the sheets were exposed to drying and calendaring processes. This means that the wash cycle that the experimental (100% cotton) sheets were exposed to was very similar to the simulations (section 3.2.4). Furthermore, the WE cycle tested is very similar to cycles processed within care facilities, either implementing HTM 01-04 onsite or

having a commercial laundry process their own sheets. When a care facility has their own sheets processed at a commercial laundry they are subjected to a WE cycle followed by drying and calendaring.

As was the case with the simulation, the number of viable cfu/25cm² from the sheets processed in a WE, were comparable to those found by Lakdawalla *et al.* (2011), who sampled from sheets laundered in a CTW. Lakdawalla *et al.* (2011) found a range of 1-3 log₍₁₀₎ cfu/100cm², whereas the present study found, 2-3 log₍₁₀₎ cfu/25cm². If the authors had reported a pre-wash spore load, it may have been possible to estimate how washing in a WE, followed by CTW might affect the number of spores surviving on processed linen.

The ribotype of the strain found in this study, before and after washing, were indistinguishable from each other and had a single difference from ribotype 001/072. It can be inferred that the spores present before washing were the same type, as those recovered after. The sampling method was limited in that the areas of faecal soiling were identified and two adjacent regions were visibly matched to be sampled pre-wash and post-wash. The post-wash samples may have had a different level of contamination before washing than the pre-wash sample, it would be unclear using this method as the sample regions were determined by the visible level of soiling. The number of spores found on

adjacent post-wash swatches might also be due to cross-contamination from elsewhere on the bed sheet, as transfer of spores within the wash has been demonstrated in the present study and in other published works (section 3.1.2. and 3.4.2).

The consequences of low levels of *C. difficile* spores surviving on laundered bed sheets, need to be considered. The spores may be transferred to the hands of anyone touching the linen and pose a transmission risk. The hand transfer rates of viable spores have not been studied in *C. difficile*, but it would be important in understanding the next step in transmission, from linen to the hands of anyone who encountered it. Rusin *et al.* (2002) investigated the transfer rates of *Serratia rubidea* and *Micrococcus luteus*, quantifying the proportion of bacterial cells transferred from surfaces to hands. Where $9.73 \log_{(10)}$ cfu of *M. luteus* were inoculated onto laundry (100% cotton swatches), $6.17 \log_{(10)}$ cfu were transferred to hands. Similarly, with an average $9.79 \log_{(10)}$ cfu of *S. rubidea* inoculated onto swatches, $4.40 \log_{(10)}$ cfu were transferred to hands. The bacterial cells were handled and sampled immediately after inoculation and were not given time to dry (Rusin *et al.*, 2002). It is not clear, whether *C. difficile* spores would be transferred in the same way or at the same rate, as cells of other bacterial species.

When assessing another spore-forming species, Lopez *et al.* (2013) quantified the transfer efficiency of *B. thuringiensis* spores, from 100% cotton swatches to hands in low and high humidity conditions. The authors demonstrated that an average transfer efficiency of 0.6% (range 0.5%-0.8%) in low humidity conditions. The transfer efficiency from cotton, was lower and also less variable, compared to non-porous surfaces, for example, acrylic which ranged from 45% to 74%. In high humidity conditions, the transfer efficiency of *B. thuringiensis* spores from cotton increased to 3.5%, with a range of 0.9% - 10%, an order of magnitude higher than in lower humidity conditions. Transfer rate from 100% cotton for *S. aureus* ranged from 0.4% to 1.9% in low humidity and 0.1% to 1.3% in high humidity.

It is feasible that the surviving spores may contribute to environmental contamination, when the sheets are used for bed-making. It has been demonstrated with bacterial cells on bed sheets that bed-making can contribute to the environmental contamination. Bed-making is highly likely to be performed after terminal or general cleaning has been undertaken, so it is feasible that spores could be dispersed in to previously cleaned rooms. If that was the case, inadequately laundered bed linen could account for a proportion of the contamination found on hospital surfaces and contribute to the 82% of non-hypervirulent infections from unknown sources.

The wash simulations (section 3.4.2) when compared with the commercial wash, showed that the overall number of spores recovered were not different to the swatches processed at the commercial site i.e. 0-14 cfu/25cm² vs 33 cfu/25 cm². The difference was in the log₍₁₀₎ reduction of spores from sheets, whereby a >6 log₍₁₀₎ reduction was demonstrated in the simulated wash compared to 0.45 log₍₁₀₎ the industrial WE cycle on the service provider site (Table 3.2). One possible reason could be variable levels of soiling in the commercial wash, as the linens were naturally contaminated and the soiling in the simulation was standardized between cycles. Another factor, could be that the simulation was performed in a laboratory setting with careful monitoring of time, temperature and the weight of fabric for standardized loading, and such precautions may not be taken under routine use in the commercial setting. To further explore the survival of *C. difficile* spores *in situ*, it would be necessary to assess the spore load on experimental (100% cotton) sheets before and after they are processed in the full cycle i.e. WE pre-wash, CTW main wash, drying and calendaring.

In summary, the *in situ* assessment of the WE infected linen wash was conducted at 90°C, which met and exceeded the minimum temperature required by HTM 01-04. The level of spore contamination before washing was lower than expected at around 1.7 log₍₁₀₎ cfu/25 cm² and the WE 90°C cycle effected only a 0.45 log₍₁₀₎ reduction. In contrast, the optimized simulations

were able to affect a $>6 \log_{(10)}$ reduction, so factors *in situ* were reducing the effectiveness of the WE cycle. The two step protocol at the commercial laundry should be studied further, to independently determine if the final processed sheets are meeting validation requirements i.e. no pathogenic bacteria. This study demonstrated that infected linen from CDI patients provided by care facilities and washed in the commercial laundry WE cycle, with drying and finishing, may be contaminated with low levels of spores. Furthermore, the evidence of spores surviving the WE cycle suggests that sheets contaminated with spores entering a WE for a pre-wash as part of the two step protocol could be present on entry to the CTW for the main wash. This would be in direct violation of HTM 01-04, which states that that infected linen must not be processed in a CTW in case of blockages, where it would then be unsafe to enter the machine. This also poses a health risk for laundry operatives, who manually sort linen entering the CTW, including 'previously infected' linen which has been pre-washed.

3.5.5 Healthcare laundry survey for care facilities

The survey demonstrated the variation in how care facilities implement the laundry policy and the different chemo-thermal conditions that are in use. Most facilities had an onsite laundry, with industrial machines and detergents, however, only 12/20 facilities provided confirmation that annual validation was performed by the company providing the equipment. This suggests that the

other 8/20 facilities may have been operating infected linen wash cycles, without validation of disinfection. This is of concern, firstly because it violates the requirement of HTM 01-04 for annual verification; but also, unrecognised problems in detergent dosing or reduction in time or temperature may then result in contaminated linen. The simulations without detergent, demonstrated the importance of detergent to significantly reducing contamination (section 3.3.3).

Furthermore, the temperature at which some facilities were washing infected linen, was well below the policy minimum requirements (Table 3.3). There were 46.1% of facilities washing below the 71°C minimum temperatures, with no information provided about additional chemical disinfection regimes to compensate for the reduced thermal disinfection e.g. Ozone. Out of the facilities washing below the policy minimum temperatures, there were 7 caring for people with CDI. This could indicate that *C. difficile* spore contamination may be found on linen processed at these facilities, if no other measures are employed and the detergents used are similar to the industrial detergent tested. The optimised simulation and the WE cycle at the commercial laundry both had residual low levels of spores, regardless of the original spore contamination level (section 3.3.3 and 3.3.5). This is supported by Mackay *et al.* (2017), who recently showed that ambulance uniform swatches inoculated with *C. difficile* spores and then washed at 30° had no log₍₁₀₎ reduction and those

washed at 60°C had 1 log₍₁₀₎ reduction. Further demonstrating the survival of *C. difficile* spores on textiles at temperatures lower than the minimum required by HTM 01-04. Furthermore, where cycle time was reduced in a single wash simulation and the spores were viewed by SEM, there were large aggregates of spores, possibly within soiling clumps. So, reducing the time held at 71°C, may cause large spore aggregates to be present on linen (Figure 3.6).

Due to restrictions at the commercial laundry, it was not possible to quantify survival through the two wash system. However, identifying survival of spores through the WE cycle (*in situ* and in simulations) is immediately relevant to the care facilities either using a commercial laundry to wash their own linen or using their own WE on site. A large proportion of the care facilities were processing their own linen in this way and those facilities also caring for CDI patients may be putting their patients at risk. Linen that is still contaminated may contribute to the environmental contamination and the high levels of asymptomatic carriage in care facilities; although the next step in linking surviving spores to environmental contamination and active CDI is yet to be demonstrated.

The care facilities must use a validated enhanced laundry process, which has the same requirements as the commercial infected linen pathway, where patients are symptomatic. Furthermore, it needs to be recognised that

asymptomatic patients can still shed large volumes of *C. difficile* spores. It has been estimated that in hospital and long-term care facilities asymptomatic carriage can reach 10% - 58% and patients may shed up to 5.9 log₍₁₀₎ *C. difficile* spores/gram faeces (Riggs *et al.*, 2007; Shaughnessy *et al.*, 2011). In addition, up to 27% of patients transferred to a care facility may become asymptomatic carriers within 6 weeks (Ponnada *et al.*, 2017).

Interestingly, CDI occurred only at those facilities which had three or more types of infectious disease present, whereas the other infectious diseases were present alone or in combination. This finding supports recent work which has shown that in long-term care facilities, drug resistant infections are part of a complex network of interactions: antibiotic use is a risk factor for primary multidrug resistant organism colonisation, which in turn increases the likelihood of colonization and infection by other multidrug resistant organisms which may be in the environment (Wang *et al.*, 2017). With increased need for antibiotics in a long-term care environment, there is increased acquisition of multidrug resistant species including CDI.

Finally, although the next step in the spread of the surviving spores to the environment is yet to be shown, it cannot be discounted. There is a case for strengthening the requirements made of care facilities in their linen processing in line with other healthcare linen providers.

3.6 Conclusions

The present investigation was a pilot study which quantified *C. difficile* spores on naturally-contaminated bed sheets before and after washing in a WE, on a commercial laundry site. It was demonstrated that the thermal disinfection conditions, described in HSG (95) 18 and updated in HTM 01-04, were inadequate to fully decontaminate linen which had been naturally contaminated with *C. difficile* spores. The detergent and additives had the greatest effect in reducing spore burden, whether by removal or possible sporicidal activity in combination with the thermal disinfection (section 4.2.3).

The EQR microbiological test for chemical disinfection is the addition of a sterile de-sized textile, which must be recovered after washing, but before drying for enumeration of bacteria; there must be no bacteria recovered.

The best practice for microbiological assessment is a >5 log reduction of a thermotolerant species of vegetative bacterium, inoculated onto test strips. If the comparison is made to these test standards, the WE cycle that was performed at the commercial laundry, failed the microbiological requirements (<1 log₍₁₀₎ reduction) due to the survival of the same ribotype of *C. difficile* spore through the WE wash cycle; even though washing was performed at a higher temperature and combined with drying and calendaring. The commercial laundry had an infected linen protocol which involved a pre-wash in the WE

followed by a second, main wash in the CTW. Where spores survived the pre-wash, transferring the linen to a CTW would be in direct violation of HSG (95) 18 (in force during the study period), which stated that infected linen must not be processed in a CTW in case of blockages; any obstruction composed of contaminated linen would make it unsafe to open the machine for repairs.

Although there is a move toward making CTWs safe for infected linen and calibrated for processing alginate bags in large quantities, this was not the case during the study period and at the study site (Department of Health, 2016a).

The presence of spores after the WE pre-wash, may also pose a health risk for laundry operatives, who manually sort linen entering the CTW main wash, including 'previously infected' linen which has been pre-washed. This may not present as an active CDI, but could be a factor in asymptomatic carriage of laundry operatives. Furthermore, a main wash cycle in a CTW, without a pre-wash has shown to be ineffective at decontaminating linen, so relying on the main cycle to render linen safe is a risk. It is necessary to follow up the present study with an assessment of the linen processed in the full cycle comprising: WE pre-wash, main wash in a CTW and high temperature drying and finishing. The whole process evaluation would elucidate whether the two wash method is effective. Until this is completed, it is unclear how many *C. difficile* spores may be present on processed linen entering hospital wards.

It is clear that care facilities using commercial laundries to wash their own linen, may be putting their patients at risk; linen provided by care facilities to the commercial laundry were processed solely in a WE with drying and finishing, to aid ease of return to the correct facility. Any facilities with symptomatic CDI or asymptomatic colonised patients, shedding spores onto linen, would be exposing their patients to inadequately processed linen and cross-contamination to other items in the wash load. The study in the commercial laundry yielded different results from the optimised simulation. In the simulations the industrial detergent performed exceptionally well and was able to achieve a $>6 \log_{(10)}$ reduction in the number of viable spores recovered.

It is possible that validation or microbiological tests used to assess the disinfection process, may not be recovering the maximum number of spores present on samples taken from sheets. Vortexing is more efficient than stomaching, when spores are inoculated in the presence of soiling.

The care facility survey highlighted the variation in how facilities meet the policy requirements and demonstrates the importance of understanding how each set of conditions, such as thermal disinfection times and temperatures and available detergents can contribute to the successful decontamination of linen.

Currently, there is no standard method to validate spore inactivation for either thermal or chemical processes, other than to test for *B. cereus* annually with destructive sampling of processed linen. It would be beneficial to have a standardised method by which all laundry processes can be compared, using an evidence-based approach to define what is the best practice for providing effectively decontaminated linen.

4 The effects of temperature, soiling and detergents on *C. difficile* spore survival

4.1 Chapter abstract

There are four factors which are associated with the effectiveness of a laundry cycle, temperature, time, agitation and chemical additives. There were low levels of *C. difficile* spores surviving the simulated and *in situ* washes; therefore, the aim of this chapter was to assess the sporicidal activity of the industrial detergent system and investigate the factors influencing the survival of *C. difficile* spores. Spore survival was quantified at 71°C and 90°C up to 20 minutes in the presence or absence of simulated soiling. The industrial detergent from the simulated healthcare wash and commercial laundry (section 3.4.3 and 3.4.5) was tested for sporicidal activity (adapted from BS EN 13704). Soiling was found to protect spores at 90°C when exposed for 5 and 10 minutes, but the effect was lost by 20 minutes, where a 3 log₍₁₀₎ reduction was demonstrated regardless of soiling. At 71°C, there was <1 log₍₁₀₎ reduction after 20 minutes, without soiling, with little opportunity to observe any protective effect of soiling. At 25°C the industrial detergent and sodium hypochlorite additive was able to achieve a 0.84 log₍₁₀₎ reduction. When the detergent was tested at 71°C, followed by the sodium hypochlorite wash additive at 60°C there was a 2.81 log₍₁₀₎ reduction; just below the 3 log₍₁₀₎ reduction required for sporicidal activity. These findings demonstrate that the combined effects of the industrial detergent and 71°C thermal disinfection, moderately reduced spore contamination in isolation from rinsing, agitation and removal from linen.

4.2 Introduction

Spores are some of the most thermotolerant biological entities known, due to their low moisture composition, complex layers including cortex, spore coat and exosporium, and the dipicolinic acid (DPA) content (Russell, 1998; Setlow, 2007; Donnelly *et al.*, 2016; Gil *et al.*, 2017). Spore thermotolerance varies between species, and also between strains within the same species even when investigations are performed under similar conditions (Kort *et al.*, 2005; Orsburn *et al.*, 2008; Luu-Thi *et al.*, 2014; den Besten *et al.*, 2017). For example, the heat resistance of spores of a given *Bacillus* spp. can be influenced by environmental factors present during sporulation, such as incubation temperature (Wells-Bennik *et al.*, 2016). Likewise, spores exhibit resistance to chemicals commonly used within the healthcare environment, such as detergents and low-level sodium hypochlorite (Maillard, 2002; Fraise *et al.*, 2015; Maillard, 2016). Validated sporicidal agents include alkylating agents and oxidising agents such as Chlorine bleach and it is not unusual for long contact times to be necessary for sporicidal activity, such as 2-4 hours (Horejsh and Kampf, 2011).

Soiling has been shown to protect bacteria from disinfectants and the damage caused by high temperatures. *B. subtilis* spores were found to resist high temperatures of 94-99°C where a 1 log₍₁₀₎ reduction in viable spores was demonstrated after 4 minutes in the presence of artificial soiling compared to 2 minutes without soiling (Diab-Elschahawi *et al.*, 2010).

The healthcare laundry policy, HTM 01-04, specifies the temperatures at which thermal disinfection may be applied, in an attempt to achieve microbiologically clean linen. These temperatures have been shown to be insufficient at inactivating *C. difficile* spores *in vitro* (Rodriguez-Palacios *et al.*, 2010; Rodriguez-Palacios and LeJeune, 2011). However, it has been shown with thermotolerant bacterial cells such as *E. faecalis* that ineffective *in vitro* inactivation does not necessarily equate to failure to decontaminate *in vivo* (Orr *et al.*, 2002). With the drive for sustainability the use of low temperature, low water and low energy wash cycles will be more widespread, which suggests chemical disinfection will become more important (European Textile Services Association, 2015). Currently the chemical disinfection process is validated by the companies that supply them and the minimum requirement is that the disinfection is as effective as or better than thermal disinfection alone. Certainly, detergents are very important to the removal of visible dirt from linen, but the importance in removing bacterial cells and spores is undetermined. In the UK, any product claiming sporicidal activity must now be externally validated by an approved laboratory (Fraise *et al.*, 2015). This chapter explores the effects of high temperatures, soiling and domestic and industrial detergents on the survival of *C. difficile* spores.

4.2.1 Moist-heat thermotolerance of *C. difficile* spores

The pertinent temperatures for this study were taken from the healthcare laundry policy, where a minimum of 71°C is required, and from the methods used at the commercial laundry previously investigated, where experimental sheets were washed at 90°C (section 3.3.5). Previous studies have focused mainly on strains of relevance to the food industry, but are still useful for understanding spore thermotolerance and the mechanism by which it inhibits viable spore recovery (Rodriguez-Palacios *et al.*, 2010; Rodriguez-Palacios and LeJeune, 2011; Hoover and Rodriguez-Palacios, 2013; Rodriguez-Palacios *et al.*, 2016). There are also infection control studies, which can be drawn on to compare the temperatures and corresponding log₍₁₀₎ reductions, particularly where thermal disinfection has been measured in isolation from other inhibitory processes (Alfa *et al.*, 2008; Alfa *et al.*, 2013; MacDonald *et al.*, 2016).

The study of *C. difficile* spore thermotolerance, in relation to prescribed temperatures, began with qualitative assessments of presence/absence of spores at various temperatures. For example, resistance was demonstrated in 95% (61/64) of *C. difficile* spore strains heated at 70°C for 20 minutes, but inhibition in 81% (52/64) of isolates heated at 85°C for 20 minutes (Wultanska *et al.*, 2004). In the first specific quantitative test of thermotolerance, 20 different strains of *C. difficile* were shown to survive at 71°C for ≥2 hours (Rodriguez-Palacios *et al.*, 2010). As can be expected, with increasing time at

71°C there were greater reductions in viable spores i.e. 30 minutes (1.3 log₍₁₀₎ cfu/ml reduction), 2 hours (2.1 log₍₁₀₎ reduction). Furthermore, 10% of strains survived 85°C for 10 minutes. In a follow up study, Rodriguez-palacios and Lejeune (2011) demonstrated that heating to 85°C for 15 minutes inactivated ≥ 6 log₍₁₀₎ cfu/ml and after 20 minutes the method was unable to recover spores.

Edwards *et al.* (2016) demonstrated strain-dependent variation in spore thermotolerance, when *C. difficile* spores were heated for 20 minutes at $\geq 70^\circ\text{C}$. There was less strain variability below 70°C, presumably because there was no significant log₍₁₀₎ reduction. At 75°C there was a 1-2 log₍₁₀₎ reduction over the three strains tested. At 80°C there was a 3-4 log₍₁₀₎ reduction. Finally, with 85°C, the maximal temperature tested, there was a 3.5-4.5 log₍₁₀₎ reduction.

Furthermore, it was shown how sub-lethal temperatures can be selective for more thermotolerant *C. difficile* ribotypes in raw meat, specifically ground beef (Rodriguez-Palacios *et al.*, 2016). With thermal inhibition curves of ribotypes, 027, 078 and ATCC 9689 heating to 85°C for up to 30 minutes, in a low-fat beef matrix, it was shown that ribotype 027 had the greatest thermotolerance, with a ~ 4.5 log₍₁₀₎ cfu/ml reduction at 20 minutes. In a study by Donnelly *et al.* (2016), spore from strains without DPA were less resistant to moist-heat than wild type *C. difficile* 630 strain, where < 1 log₍₁₀₎ reduction was

seen after 15 minutes at 80°C. In the DPA⁺ strains a ~4 log₍₁₀₎ reduction was demonstrated by 15 minutes at 80°C.

There are disparate estimates for the number of *C. difficile* spores recovered after heating to 85°C specifically, ranging from no viable spores (6 log₍₁₀₎ reduction) after 20 minutes to 3.5 log₍₁₀₎ cfu/ml viable spores after 30 minutes (Rodriguez-Palacios and LeJeune, 2011; Edwards *et al.*, 2016). The picture is complicated by the use of different strains and ribotypes. For example, ribotype 027 appears to be very resistant to moist-heat treatment and others such as ATCC 9689, and animal recovered strains, are more sensitive (Rodriguez-Palacios *et al.*, 2016). With regard to disinfection temperatures, all agree 70-75°C can have a significant effect on viability of spores, after long treatment times of up to 2 hours a 2 log₍₁₀₎ reduction could be expected. This equates to inactivating 99% of viable spores in the original spore load, but does depend on the level of contamination. Macdonald *et al.* (2016) described a decontamination cycle in a bed pan washer which reached 91°C for 1 minute during which *C. difficile* spores were exposed to a faecal emulsion within a cryovial. Again, there was a large range in the number viable spores recovered, demonstrating a 1-4 log₍₁₀₎ reduction in spores from the original 7 log₍₁₀₎ cfu/ml inoculum. Therefore, 91°C for 1 minute left between 3-6 log₍₁₀₎ cfu/ml viable spores, when thermal disinfection alone was tested. This combination is

comparable to wash cycles in domestic washing machines where the temperature is reached but not held for any length of time.

The presence of soiling may also affect the resistance of cells and spores, by preventing contact with chemicals and reducing the effects of high temperatures. Diab-Elschahawi *et al.* (2010) investigated artificial soiling using a wheat and egg suspension, exploring the effect on thermotolerance in enterococci vegetative cells and *B. subtilis* spores. The presence of artificial soil in the test suspension significantly reduced the effectiveness of moist-heat on both cells and spores. *B. subtilis* spores were exposed to temperatures up to 99°C for 10 minutes in the presence and absence of the artificial soil. Unsurprisingly the spores exhibited greater thermotolerance than enterococci cells and at 94°C it took almost three minutes to achieve a 1 log₍₁₀₎ reduction in *B. subtilis* spores when heat-treated in PBS, compared to just over four minutes when heat-treated in artificial soil. The *B. subtilis* spores survived for significantly longer time periods in the presence of soiling at very high temperatures, between 94°C and 99°C.

In summary, the thermotolerance of spores from a given strain of *C. difficile*, may vary dramatically and the effects of simulated soiling on the decimal reduction time of *B. subtilis* spores hint at a protective effect at high temperatures. The effect of soiling has not been considered in *C. difficile* spores,

but may also contribute to even greater thermotolerance than expected.

Therefore, the sporicidal capacity of laundry detergent must be considered, in combination with thermal disinfection and with soiling, to understand how each contributes to the inactivation of *C. difficile* spores.

4.2.2 Domestic and Industrial detergents and decontamination of *C. difficile* spores

In combination with high temperatures, chemistry is another critically important factor that influences the efficacy of laundering processes. There are many different detergents on the market; Halo[®] commercial and Halo[®] non-bio both contain Hygenilac, which is claimed to decontaminate *C. difficile* spores during a low temperature wash (30°C). Although the data is not available, there is a claim of meeting validation requirements of BS EN 13704: Chemical Disinfectants – Quantitative Suspension Test for the Evaluation of Sporicidal Activity of Chemical Disinfectants Used in Food, Industrial, Domestic and Institutional Areas (British Standards Institution, 2002). There are many industrial detergents and detergent systems which are available to commercial laundry providers and care facilities choosing to perform enhanced process laundering onsite. The laundry detergents used can be made up of many different components, making it very difficult to estimate the impact of a certain compound or to compare different studies.

However, there are three major groups of ingredients that may determine the overall antimicrobial or sporicidal efficacy of detergents: surfactants and oxidising agents (Bockmühl, 2017). Surfactants are an important component of detergents and account for the basic cleaning efficacy and removing hydrophobic soil. Honisch *et al.* (2016) investigated laundry processes with and without detergents and found little or no effects when comparing the remaining microbial load on artificially contaminated swatches after laundering in a domestic washing machine. *S. aureus* cells (and less so *Enterococcus hirae*) exhibited a considerably higher $\log_{(10)}$ reduction when washed with detergent compared to water alone. It has been suggested that this is due to better removal of bacterial cells from the fabric, since suspension tests show no antimicrobial effects when exposed to the detergents (Brands *et al.* 2016).

Furthermore, when laundry detergents with and without peroxide (activated oxygen bleach (AOB)) were compared, the additional $\log_{(10)}$ reduction due to peroxide was shown to be 2-6 $\log_{(10)}$. These values may vary with the other test conditions e.g. temperature, time and the tested organisms (Honisch *et al.* 2014). Furthermore, chlorine bleach can enhance the antimicrobial efficacy of laundering by 3 - 4 $\log_{(10)}$, even at low temperatures (Walter & Schillinger, 1975; Christian, 1983; Blaser *et al.*, 1984; Smith *et al.*, 1987).

Environmental detergents (non-chlorine containing) have been shown to enhance sporulation in *C. difficile* cells cultured in faeces with sub-inhibitory concentrations of one commonly used hospital cleaning agent (Wilcox and Fawley, 2000; Fawley *et al.*, 2007). Types of environmental disinfectant such as quaternary ammonium and phenolic compounds have limited sporicidal activity and alcohol has no sporicidal activity (McDonnell and Russell, 1999; Setlow, 2006; Maillard, 2011; Sattar and Maillard, 2013). There are several compounds which are active against *C. difficile* spores; for example, chlorine bleach and other oxidising compounds, which are likely to inactivate spores by damaging the external layers around the core, possibly the inner membrane, which then ruptures during outgrowth (Loshon *et al.*, 2001; Genest *et al.*, 2002; Young & Setlow, 2003; Leggett *et al.*, 2012; Maillard, 2016).

Perez *et al.* (2005) demonstrated the variation in effectiveness of oxidising compounds for inactivating *C. difficile* spores. The microbicides tested were domestic bleach with free-chlorine (FC) levels of 1000, 3000, and 5000 mg/L; an accelerated hydrogen peroxide (AHP)-based product with 70,000 mg/L hydrogen peroxide (Virox STF); chlorine dioxide (600 mg/L FC); and acidified domestic bleach (5000 mg/L FC). The acidified bleach, hydrogen peroxide (Virox STF), and regular bleach (3000-5000 mg/L FC) could inactivate 5 log₍₁₀₎ *C. difficile* spores on hard environmental surfaces (stainless-steel) in ~10-15

minutes under ambient conditions. Regular bleach, 1000 mg/L FC took up to 25 minutes to achieve a 5 log₍₁₀₎ reduction.

Büttgen *et al.* (2008) assessed the sporicidal activity of 1% glutaraldehyde and demonstrated a <2 log₍₁₀₎ reduction in *C. difficile* spores after 15 minutes. When used at 2% glutaraldehyde may inactivate *C. difficile* spores within 10 minutes (Dyas & Das, 1985).

Hospital and laboratory disinfectant agents have been shown to vary dramatically in their sporicidal activity against *C. difficile* spores and there is some debate over efficacy (Fawley *et al.*, 2007; Vohra and Poxton, 2011; Ali *et al.*, 2016). For example, Virkon has been shown to have a sporicidal effect at 1, 10 and 20 minutes against spores in the absence of soiling present; however, the main active compound, potassium peroxymonosulfate, was inhibited when simulated soiling was present (Vohra and Poxton, 2011). Where BSA at 0.27%, was used to simulate organic matter, the sporicidal effect of Virkon was significantly reduced from 1.2 to 0.2 log₍₁₀₎ reduction of spores after 10 minutes. This was the also the case for Trigene Advance (alkyl dimethyl benzyl ammonium chloride, didecyl dimethyl ammonium chloride) which demonstrated a lower log₍₁₀₎ reduction in the presence of simulated organic matter (1.7 vs 0.5 log₍₁₀₎ reduction) and Microsol 3+ (didecyl dimethyl ammonium chloride) which had 1.5 vs 0.7 log₍₁₀₎ reduction.

Sudhaus (2014) treated *B. cereus* strains with various concentrations of a representative peracetic acid-based disinfectant at three temperatures (10, 15 and 20°C), with protein load and with different exposure times (5, 30 and 60 minutes). The strains used exhibited a great variability in their resistance to peracetic acid and the addition of 10% BSA had no effect on the log₍₁₀₎ reduction achieved for the majority of temperature and time combinations. However, peracetic acid-based disinfectants, have demonstrated poor stability, with a significant reduction from 1500ppm to between 50-800ppm after short-term storage. Furthermore, where the peracetic acid concentration falls to <600ppm the sporicidal activity against *C. difficile* spores may be significantly reduced (Cadnum *et al.*, 2017).

To assess the sporicidal activity of a given compound or combination of agents, the European standard BS EN 13704:2002 was used to develop a sporicidal test method, which could test for efficacy against *C. difficile* spores (British Standards Institution, 2002). Issues have been highlighted with the requirements of BS EN 13704, including the use of surrogate species such as non-pathogenic spore former *B. subtilis*, unrealistic exposure times of 5 and 60 minutes and test conditions which were not relevant to the practical use of the solutions or compounds being tested (Fraise, 2011). In light of these issues, changes were made to include the testing of *C. difficile* spores, at the time and temperatures relevant to the laundry industry (section 3.1.1).

More recently an updated protocol by Fraise *et al.* (2015) describes the production of spore suspensions for the testing of the sporicidal activity of chemical disinfectants. The authors collaborated with PHE to establish a new test protocol using *C. difficile* strain NCTC 11209 and called for a $>5 \log_{(10)}$ cfu/ml reduction to be accredited as a sporicidal agent. The test method also considers the issues of BS EN 13704:2002, by proposing realistic contact times as well as the higher $\log_{(10)}$ reduction which must be met to keep healthcare environments safe for patients.

4.2.3 In summary

Bacterial spores are recalcitrant to decontamination, protecting the DNA of the vegetative cell and facilitating resistance to challenging conditions. The NHS healthcare laundry policy describes conditions, such as high temperature, for minimum times and the use of chemical additives, all of which may damage the spore or remove it, with the aim of decontamination and rendering linen safe. The accumulative success of these conditions has been shown to be variable, dependent on the specifics of the whole wash process (section 3.3.3. and 3.3.4.). The temperature and time held are the primary method of disinfection, but *in vitro* studies show that spores can survive the 71°C minimum temperature for longer than 2 hours. The detergent may act as a surfactant by removing spores, but may also have some additional sporicidal activity, particularly chlorine bleach and peracetic acid. The level of soiling present in a

wash may also have an effect, so it is not clear how the each of the factors are contributing to the survival of *C. difficile* spores.

4.2.4 Scope of the work

4.2.4.1 Aim

The aim of this investigation was to compare the effects of heat, detergent and soiling on the inactivation of *C. difficile* spores *in vitro*.

4.2.4.2 Objectives

1. To quantify the survival of *C. difficile* spores exposed to the temperature advised in the healthcare laundry policy HTM 01- 04.
2. To assess the effect of soiling on the survival of *C. difficile* spores at the temperature advised in the healthcare laundry policy HTM 01- 04.
3. To quantify the sporicidal activity of an industrial detergent and a reference domestic detergent on the survival of *C. difficile* spores at contact times found in a healthcare wash.

4.3 Methods

4.3.1 Moist-heat thermotolerance of *C. difficile* spores

The spore suspensions used for this investigation were generated using the non-heat exposed method (section 2.2.5.3.). *C. difficile* NCTC 11209 spores in MRD (1 ml) were exposed to 71°C (5 log₍₁₀₎ cfu/ml) or 90°C (6 log₍₁₀₎ cfu/ml) for 3, 5, 10 and 20 minutes in a rotating water bath at 100rpm. The centrifuge tube was added to the water with a control tube containing 1 ml of MRD and a thermometer sealed in the tube. The test time began when the control tube reached the test temperature. The test suspension was vortexed at the end of the exposure time, then immediately either serially diluted or vacuum filtered. For 71°C tests, 0.1ml of each dilution was spread-plated in duplicate onto pre-reduced BHIS/T. For 90°C, all tests were serially diluted and plated, additionally, at 10 and 20 minutes aliquots of 0.1ml were immediately vacuum filtered (section 3.2.4.B.) and membranes transferred to pre-reduced BHIS/T plates. A validation control was serially diluted and spread-plated on BHIS/T from the test suspensions, to verify the concentration of the original inoculum and ensure growth conditions were anaerobic. All plates were incubated anaerobically for 48 hours at 37°C.

4.3.2 Simulated soiling and *C. difficile* spore thermotolerance

BSA was used to simulate the effect of soiling on spore survival at the temperatures used for thermal disinfection. To simulate dirty conditions a BSA master solution (30g/L) was filter sterilized and added to the spore suspension to give a final concentration of 3g/L. BSA (0.1ml) was added to a *C. difficile* NCTC 11209 spore suspension sample (0.9ml), the test mix was vortexed, added to the water bath and timing began when an identical 1ml of MRD reached the test temperature. The test temperatures were 71°C (5 log₍₁₀₎ cfu/ml) and 90°C (6 log₍₁₀₎ cfu/ml) for 3, 5, 10 and 20 minutes, in a rotating water bath at 100rpm. A validation control was serially diluted and spread-plated on pre-reduced BHIS/T from the test suspensions, to verify the concentration of the original inoculum and ensure growth conditions were anaerobic. The viable spores were quantified as previously described (section 4.2.1).

4.3.3 Assessing the sporicidal activity of a reference domestic detergent and industrial detergent system

The sporicidal activity of domestic and industrial detergents was quantified using methods adapted from: BS EN 13704:2002 Quantitative Suspension Test for the Evaluation of Sporicidal Activity of Chemical Disinfectants Used in Food, Industrial, Domestic and Institutional Areas. The method of neutralisation chosen was vacuum filtration; a validation control was performed to confirm neutralisation. For the validation test a 3 log₍₁₀₎ cfu/ml *C. difficile* NCTC 11209

spore suspension was prepared (section 2.2.5.3). The vacuum filter unit was prepared with a black filter membrane and 50 ml of SDW, then 0.1ml of detergent (Table 4.1) or SDW control was added. The fluid was filtered, then 150 ml of SDW was added and filtered. Finally, 0.1 ml of the spore suspension was added to a further 50ml SDW and filtered. The membrane was placed onto pre-reduced BHIS/T and incubated anaerobically at 37°C for 48 hours. The number of viable spores recovered were compared between SDW control detergent filtration plates and an enumeration control of the test suspension.

4.3.3.1 Sporocidal activity of the reference detergent

For assessing sporocidal activity of the reference domestic detergent (Ref detergent A, SDC, UK) 0.1ml of *C. difficile* NCTC 11209 spores in MRD and 0.1ml BSA (3g/L) were vortexed and placed in a water bath for 2 minutes. The detergent was made up to the test concentration (Table 4.1); a 1 ml aliquot of detergent was pre-heated to test temperature for 10 minutes, with 0.8ml added to the pre-heated BSA/spore mixture at the start of the test time. All other reagents were kept at 25°C, room temperature. The test temperatures were 25°C, 71°C and 90°C (3, 3 and 6 log₍₁₀₎ cfu/ml respectively) and test times were 3, 5, 10 and 20 minutes. There was no pre-heating required for the 25°C test time as all reagents were maintained at 25°C in a water bath for the test.

At the end of the test time, the test mixture was vortexed and the viable spores were quantified and neutralised by vacuum filtration as previously

described (section 3.2.4) There were two controls being tested simultaneously, an enumeration control where serial dilutions of the spore suspension used in the investigation were plated (0.1 ml) in duplicate on to BHIS/T agar plates to assess the original inoculum in each test and SDW positive controls.

4.3.3.2 Sporicidal activity of the industrial detergent system supplied for the industrial laundry cycle simulations.

The industrial detergent was supplied as part of a validated detergent system, which is commonly used across the industrial healthcare laundering sector in the UK, including at the industrial laundry assessed in the previous chapter (section 3.2.5). The supplier set the concentration of each of the detergent components for the simulations, which were then scaled down and made up just before use (Table 4.1).

C. difficile 11209 spore suspension (0.9ml) and 0.1ml BSA (final concentration 3g/L) were added together, vortexed and placed in a water bath at 71°C for 2 minutes. The industrial detergent was made up into solution just prior to use; a 1 ml aliquot of industrial detergent solution (Table 4.1), was pre-heated to 71°C for 10 minutes, with 0.8ml added to the pre-heated BSA/spore mixture at the start of the test time. The test mixture was vortexed and placed in a rotating water bath (100 rpm) at 71°C for 11 minutes. At the end of the test time, the mixture was vortexed and then centrifuged at 5000 rpm for 1 minute. The bleach additive was made up just prior to use; a 1.5 ml aliquot was pre-

heated to 60°C for 10 minutes in a second water bath. After the test mixture was centrifuged, the supernatant was discarded and 1ml of pre-heated bleach additive was added. The test mixture was vortexed to resuspend the pellet and heated 60°C at 100 rpm, for 5 minutes. At the end of the test time the test mixture was vortexed; the detergent was neutralised by vacuum filtration and the viable spores were quantified as previously described (section 3.2.4). The controls were, a dilution control with 0.1ml plated in duplicate on to pre-reduced BHIS/T to assess the original inoculum in each test. In addition, each temperature and time period had SDW positive controls to provide validation of the test protocol. The detergent was considered to have passed the test for sporicidal activity if a $>3 \log_{(10)}$ reduction was demonstrated according to BS EN 13704:2002 Quantitative Suspension Test for the Evaluation of Sporicidal Activity of Chemical Disinfectants Used in Food, Industrial, Domestic and Institutional Areas.

Table 4.1. Reference domestic detergent (SDC) and Industrial detergent (Washing Systems) components and amount used for *in vitro* tests as recommended by manufacturers. Each detergent made up in SDW, to maintain agent:water ratio provided in wash cycle (1.25x concentration to account for dilution with spore/BSA test suspension).

Detergent type	Composition	Amount of agent*	Stage	Temp. (°C)
Reference	Detergent	1.04g:50 ml	Initial	
	Sodium perborate	0.27g:50 ml	Initial	25, 71 or 90
	TAED**	0.04g:50 ml	Initial	
Industrial	Detergent Mix part 1	150µl:43ml	Initial	
	Detergent Mix part 2	37.5µl:43ml	Initial	71
	Detergent Mix part 3	37.5µl:43ml	Initial	
	Sodium hypochlorite-based bleach***	37.5µl:25.5ml	After 1 st Wash	60

*Ratio amount of product added to SDW e.g. 1.04g added to 50ml of SDW

**Tetraacetythylenediamine, peroxide bleach activator.

***Peracetic acid sour rinse to neutralize alkaline bleach rinse, not tested in this investigation.

4.3.4 Statistical analyses

All statistical analyses were performed with IBM SPSS v. 22. The significance value for all tests was set at $p \leq 0.05$. The Shapiro-Wilk test for the normality of the distribution and Levene's test for equality of variance were used to determine whether data could be analysed by t-test or independent ANOVA (with Tukey's post-hoc tests). Where the assumption of normal distribution of the data was violated, the non-parametric Mann-Whitney *U* test or Kruskal-Wallis H test was performed, with *post-hoc* multiple comparisons and adjusted significance. Each investigation was repeated in triplicate on two separate occasions, unless otherwise stated.

4.4 Results

4.4.1 Moist-heat thermotolerance of *C. difficile* spores and the protective effect of simulated soiling

After treatment of spores at 90°C the colonies grown on BHIS/T agar showed a marked difference in the range of colony sizes (Figure 4.1 A). Smaller colonies were seen growing adjacent to large colonies. The colonies from the enumeration control show expected colony sizes, i.e. where there were colonies closer together they were smaller and when there were fewer colonies, each was able to grow much bigger. The suspensions exposed to 71°C for 20 minutes

showed no difference in colony sizes compared to the plates for the enumeration controls.

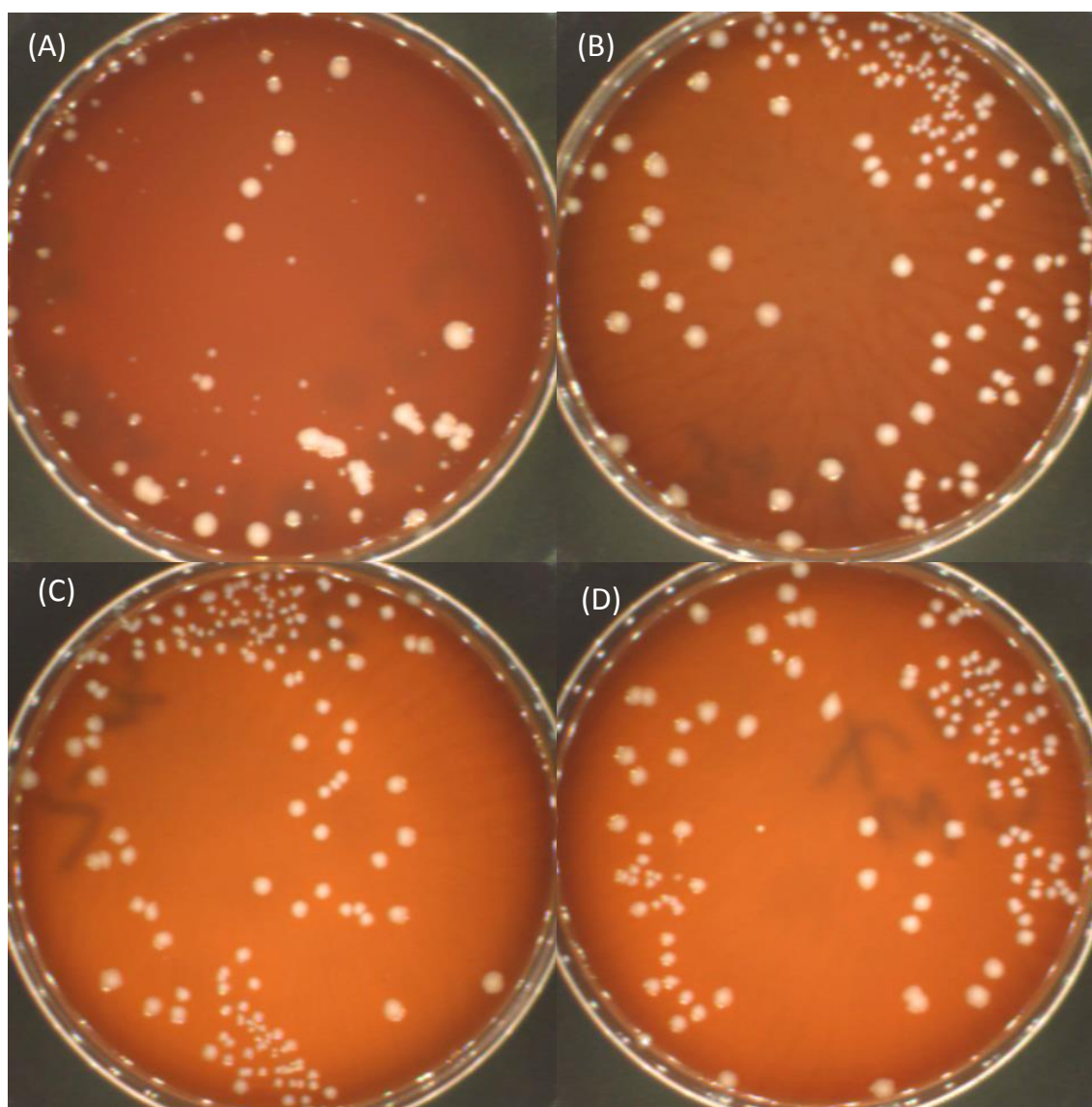


Figure 4.1 *C. difficile* colonies on BHIS/T agar (A) 90°C treated for 3 minutes, (B) enumeration control (C) 71°C treated for 3 minutes, (D) enumeration control.

Comparison by ANOVA demonstrated there were significant differences in the number of viable spores recovered at 71°C in tests without simulated soiling (Figure 4.2) ($f=16.986$, $p\leq 0.05$). In the absence of simulated soiling, there

were significantly fewer spores recovered after 20 minutes ($4.73 \log_{(10)} \text{ cfu/ml}$) compared to 0 minutes ($5.1 \log_{(10)} \text{ cfu/ml}$, $p \leq 0.05$), 3 minutes ($5.12 \log_{(10)} \text{ cfu/ml}$, $p \leq 0.05$) and 5 minutes ($5.02 \log_{(10)} \text{ cfu/ml}$, $p \leq 0.05$). Between 10 minutes and 20 minutes there was no difference in number of spores recovered (4.78 vs $4.73 \log_{(10)} \text{ cfu/ml}$, $p > 0.05$). In contrast, there was no significant difference at 0 minutes and 20 minutes in the presence of soiling, where $5.05 \log_{(10)} \text{ cfu/ml}$ spores were recovered at both test times ($\chi^2=4.474$, $p > 0.05$). When directly comparing the recovery when soiling was present or absent for each time point by t-test, there were no statistically significant differences in the number of spores recovered at any time point ($p > 0.05$).

When treated at 90°C , simulated soiling had no significant effect on the number spores recovered after 3 minutes, with $3.84 \log_{(10)} \text{ cfu/ml}$ without soiling and $3.86 \log_{(10)} \text{ cfu/ml}$ with soiling ($p > 0.05$) (Figure 4.2). However, the number of recovered spores was significantly higher in the presence of soiling after 5 minutes (3.49 vs $3.94 \log_{(10)} \text{ cfu/ml}$, $p \leq 0.05$) and 10 minutes (2.69 vs $3.90 \log_{(10)} \text{ cfu/ml}$, $p \leq 0.05$). In the final 20 minute sample, there was no significant difference in number of spores recovered whether soiling was absent or present, $2.75 \log_{(10)} \text{ cfu/ml}$ and $2.86 \log_{(10)} \text{ cfu/ml}$, respectively ($p > 0.05$) (Figure 4.2).

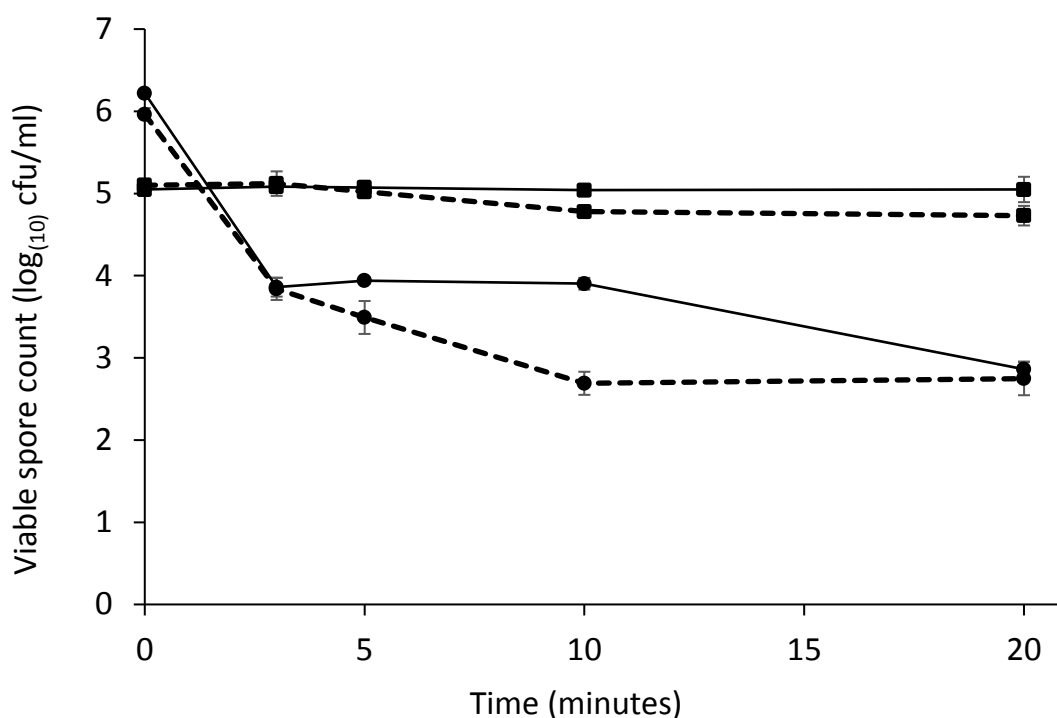


Figure 4.2. Moist heat thermotolerance of *C. difficile* (11209) spores at 71°C (■) and 90°C (●) in the presence (solid line) and absence (hatched line) of soiling (Mean \pm SE, $n=6$).

4.4.2 Assessing the sporicidal activity of a reference domestic detergent and an industrial detergent system

4.4.2.1 Reference domestic detergent

The reference domestic detergent data showed an insignificant reduction in the number of viable spores recovered at 25°C, after 3 minutes (0.27 log₁₀ reduction, $p>0.05$). There were no significant differences up to and including 20 minutes exposure ($\chi^2=28.613$, $p>0.05$) (Figure 4.3). There were significant differences in the numbers of viable spores recovered at 71°C after exposure to

the domestic detergent in the presence of soiling ($\chi^2=43.844$, $p\leq 0.05$) (Figure 4.3). There were significantly fewer spores recovered after 20 minutes (2.34 $\log_{(10)}$ cfu/ml) compared to 0 minutes (3.97 $\log_{(10)}$ cfu/ml, $p\leq 0.05$), 3 minutes (3.00 $\log_{(10)}$ cfu/ml, $p\leq 0.05$) and 5 minutes (3.00 $\log_{(10)}$ cfu/ml, $p\leq 0.05$). In brief, by 20 minutes there was a 1.43 $\log_{(10)}$ cfu/ml reduction. Furthermore, a 1.28 $\log_{(10)}$ cfu/ml reduction was seen at 10 minutes, which was significantly different from the 0 minutes sample ($p\leq 0.05$). Furthermore, between 10 minutes and 20 minutes there was no difference in number of spores recovered (2.69 vs 2.34 $\log_{(10)}$ cfu/ml, $p>0.05$).

There was a significant difference in the number of spores recovered when the reference domestic detergent was tested at 90°C ($\chi^2=33.421$, $p\leq 0.05$). All time points showed a significant $\log_{(10)}$ reduction in the number of spores recovered, compared to the 0 minutes enumeration control ($p\leq 0.05$), but were not significantly different from each other ($p>0.05$) (Figure 4.3). With a 3.06 $\log_{(10)}$ reduction after 3 minutes, the domestic detergent would be considered to have a sporicidal effect at 90°C, according to BS EN 13704 sporicidal test standard. In addition, 5, 10 and 20 would all be considered a pass but not by the more recently published test standard by Fraise *et al.* (2015).

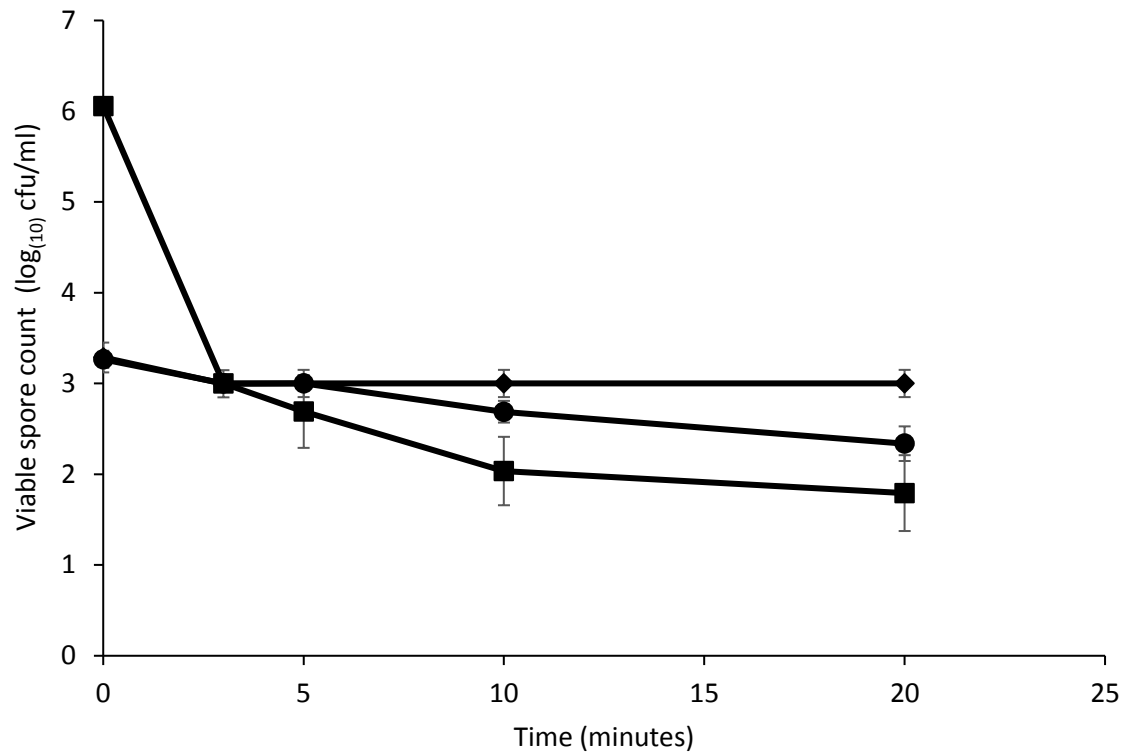


Figure 4.3. *C. difficile* spore survival in reference detergent at 25°C (◆), 71°C (●) and 90°C (■) (Mean ± SE, $n=6$).

In the validation of filtration test there was no significant difference in the number of spores recovered between the original inoculum compared to SDW control reference detergent and industrial detergent samples ($\chi^2=4.543$, $p>0.05$) (Table 4.2).

Table 4.2. Validation of filtration method of neutralisation and the effect on survival of *C. difficile* spores (as described in BS EN 13704).

Test Condition	Mean spores (Log ₍₁₀₎ cfu/ml)	Standard error of the mean
Enumeration of test suspensions	2.98	0.04
SDW	2.91	0.10
SDC reference domestic detergent	2.97	0.04
Industrial detergent system	2.92	0.03

4.4.2.2 Industrial detergent system

The combined sporicidal effect of the industrial detergent and bleach at the healthcare wash operating temperatures was significant ($t=27.053$, $p\leq 0.05$).

When used at the recommended concentration and temperature, there was a 2.81 log₍₁₀₎ cfu/ml reduction in number of spores recovered, from 5.95 log₍₁₀₎ cfu/ml to 3.14 log₍₁₀₎ cfu/ml (Figure 4.4). However, this was not a 'pass' using the 3 log₍₁₀₎ reduction threshold to be classed as sporicidal, as described in BS EN 13704. Similarly, using the more stringent >5 log₍₁₀₎ reduction threshold suggested by Fraise *et al.* (2015), the detergent and bleach combined would fail. In the absence of heat, the industrial detergent and bleach exposures had a small but significant sporicidal effect and resulted in a 0.84 log₍₁₀₎ reduction in spores compared to the enumeration control ($U=64.00$, $p\leq 0.05$) (Table 4.3). The detergent and bleach in combination at 25°C would not be considered

sporicidal, as it was unable to achieve the $>3 \log_{(10)}$ reduction required by BS EN 13704.

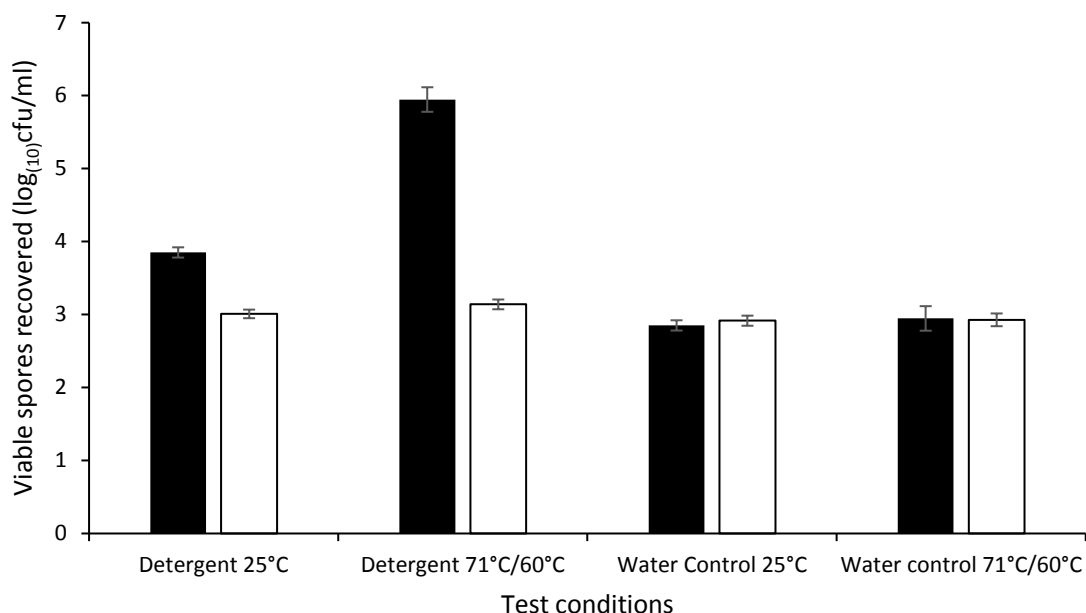


Figure 4.4. *C. difficile* spores (11209) after *in vitro* exposure to industrial detergent stage and bleach stage at industrial wash cycle concentrations and test times and SDW controls. Original inoculum (■) and post-exposure to test condition (□) (Mean \pm SE, $n=4$).

Process controls in which the spores were exposed to the test temperature and SDW, showed no significant reduction in viable spores recovered compared to the enumeration control ($t=-0.162$, $p>0.05$). As expected, there was no significant difference in the number of viable spores recovered after exposure to SDW at room temperature compared to the enumeration control, although there was a slight increase in the average number of spores recovered ($U=23.00$, $p>0.05$).

4.5 Discussion

4.5.1 Moist-heat thermotolerance of *C. difficile* spores and the protective effect of simulated soiling

The aim was to compare the effects of heat, detergent and soiling on the inactivation of *C. difficile* spores *in vitro*, at temperatures pertinent to the healthcare laundry policy and used in industry. It was demonstrated that the spores from *C. difficile* NCTC 11209 exhibited resistance to the minimum temperature for thermal disinfection, where $<1 \log_{(10)}$ reduction was seen after 20 minutes (Figure 4.2). The 20 minute test time was longer than the thermal disinfection stage of a healthcare wash cycle (≥ 3 minutes + 8 minutes minimum). The spores had a greater thermotolerant than expected, given the findings of Rodriguez-palacios *et al.* (2010), who demonstrated a $1.8 \log_{(10)}$ reduction at 30 minutes and $2.1 \log_{(10)}$ reduction after 2 hours at 71°C . The present study provides further evidence that the thermal disinfection temperature alone is unable to provide complete decontamination; where $6 \log_{(10)}$ cfu/ml spores are present, treating by thermal disinfection at 71°C for 20 minutes, will leave $\geq 5 \log_{(10)}$ cfu/ml viable spores.

The present study is the first to assess the effects of simulated soil on the thermotolerance of *C. difficile* spores at 71°C and at 90°C . There was no observable protective effect of soiling when spores were treated at 71°C , due to

the insignificant $\log_{(10)}$ reduction seen when soiling was not present. Conversely, at 90°C a protective effect was shown, which agrees with Diab-Elschahawi *et al.* (2010), who report the protective effect of artificial soiling for *B. subtilis* spores at 94°C. Numerous strains of *B. subtilis* exhibited a 1 $\log_{(10)}$ reduction at 4 minutes in the presence of soil, in comparison to 2 minutes without soiling. *B. subtilis* spores are known for being highly thermotolerant and have been used as a surrogate for *C. difficile* spores to test sporicidal activity (Diab-Elschahawi *et al.*, 2010; Fraise, 2011).

The higher test temperature of 90°C, without soiling, demonstrated an average 3.21 $\log_{(10)}$ reduction after 20 minutes (Figure 4.2). This contrasts with Rodriguez-palacios and Lejeune (2011), who found that 20 minutes at 85°C was sufficient to produce a >5 $\log_{(10)}$ reduction in viable spores. The differences could be accounted for in part by considering the different strains used and the methods of recovery (Rodriguez-Palacios *et al.*, 2016).

There was an unexpected difference in the size of colonies which suggested there may have been three outcomes for spores present in the test suspension (Figure 4.1). The first outcome was inactivation, which accounted for the largest proportion (>99.9%) of the original inoculum; Second, of those able to germinate and outgrow, some were able to do this immediately, whereas others represent a third category of heat-damaged spores which experienced a

delay in germination or outgrowth. To further explore the 'three categories' theory, the heat treatments could be repeated and treated spore suspensions could be enriched over 12 hours to see if the recovery rates are different. Alternatively, 0.1ml samples could have been transferred to a taurocholate rich liquid medium, with the light absorbance read over a 48 hour anaerobic incubation time to assess germination (reduction in OD₆₀₀) and outgrowth/proliferation (increase in OD₆₀₀). This could be compared to the pattern of OD₆₀₀ of a non-heated control incubated alongside the previously heated samples.

This theory is supported by Rodriguez-Palacios *et al.* (2010), who reported the reversal of the inhibitory effect of heating to 85°C, when *C. difficile* spores were incubated in BHI broth for 7 hours before enumeration. The evidence from the present study and Rodriguez-Palacios *et al.* (2010) imply a sub-population of heat-damaged but still viable spores. It can be hypothesised that there may have been supportive nutrients in the media used in the present study, which supported repair and recovery of germinated spores and permitted outgrowth of vegetative cells (Rodriguez-Palacios *et al.*, 2010). Similar patterns have been reported for *Bacillus* spp. which showed that different media, can affect viable spore recovery rates after moist heat exposure (Gonzalez *et al.*, 1995; Cazemier *et al.*, 2001). Different media was shown to either enhance or inhibit growth and therefore the calculated rates of survival

and thermotolerance. It has also been suggested that in *Bacillus* spp., moist heat may denature one or more vital proteins which are necessary for outgrowth of the germinated spores (Coleman *et al.*, 2010). This may also be the case for *C. difficile* spores, and could explain the lag time seen for some colonies between germination and outgrowth (Gil *et al.*, 2017). Alternatively, Setlow (2007) discussed the DNA repair strategies of *Bacillus* spp. – showing that spores underwent a period of DNA repair during germination. This repair phase, could explain the heterogeneous germination and/or outgrowth shown at 90°C if the moist-heat had caused DNA damage.

In summary, the thermotolerance of *C. difficile* spores at 71°C for 20 minutes, suggests other factors such as sporicidal and/or removal effects of the laundry chemistry may be the main factors in achieving effective decontamination of spores in industrial laundries. Thermal disinfection is not effective in isolation, however, using a high temperature wash (90°C) for greater than 10 minutes can significantly reduce spore contamination. The disadvantage being that it would require a large energy input and be prohibitively costly. Therefore, using sporicidal detergents and additives or facilitating removal of spores, could provide an answer to reducing temperatures, and achieving microbiologically safe linen.

4.5.2 Assessing the sporicidal activity of a reference domestic detergent and industrial detergent system

The SDC reference domestic detergent exhibited a minor sporicidal effect when used at 71°C for 20 minutes, but failed the $>3 \log_{(10)}$ threshold reduction of the BS EN 13704 sporicidal test standard and the higher $>5 \log_{(10)}$ reduction threshold of the published test standard by Fraise *et al.* (2015) (British Standards Institution, 2002; Fraise, 2011; Fraise *et al.*, 2015). When *C. difficile* spores were exposed to the components of an industrial detergent system commonly used in industry, there was a significant reduction in viable spores, however, this was not a 'pass' using the $>3 \log_{(10)}$ reduction threshold described in BS EN 13704:2002 Quantitative Suspension Test for the Evaluation of Sporicidal Activity of Chemical Disinfectants Used in Food, Industrial, Domestic and Institutional Areas (Table 4.3). The average $\log_{(10)}$ reduction was close to the threshold ($2.81 \log_{(10)}$), so with a greater number of replicates the $3 \log_{(10)}$ reduction may be achieved by the industrial detergent. The industrial detergent and bleach stages together, failed to achieve the $>5 \log_{(10)}$ reduction threshold suggested by Fraise *et al.* (2015).

In the absence of heat, the industrial detergent and bleach exposures resulted in a small but significant, reduction in spores compared to the SDW controls ($p \leq 0.05$) (Table 4.3). The industrial detergent and bleach in combination at 25°C would not be considered sporicidal, under either threshold,

but is useful in understanding what factors are at work in an industrial wash.

When the effect of the thermal disinfection temperature (71°C) is combined with the industrial detergent and bleach, far more spores were inactivated than using either alone. This suggests that the detergent and/or bleach are working with the temperature to inactivate spores. It has been demonstrated that mildly lethal treatment with a variety of agents may sensitize the surviving spores to a subsequent treatment, such as moist-heat, for which an undamaged inner membrane may be required for full resistance (Cortezzo *et al.*, 2004). The converse may also be true, where the heating of spores denatures protective proteins, which then allows the test compounds to reach the inner layers of the spore which may be more sensitive (Coleman *et al.*, 2010).

The validation controls with SDW demonstrated that there was no loss of spores due to the method, such as spores being lost during the centrifuging and removal of supernatant. Furthermore, process controls where spores were exposed to the test temperature and SDW, showed no significant reduction in viable spores recovered compared to the enumeration control ($p>0.05$). These findings provide further evidence that the thermal disinfection alone is ineffective at inactivating spores (Figure 4.3).

The sporicidal effect of the industrial detergent system may be due in part to the addition of the bleach stage. Recently, Edwards *et al.* (2016)

demonstrated that spores have high levels of resistance to ethanol, hydrogen peroxide and chloroform. Conversely, *in vitro* studies have shown that regular bleach (5000 ppm) and hydrogen peroxide (700 ppm) inactivate 6 log₍₁₀₎ spores in 10 minutes (Barbut, 2015). Furthermore, a study that evaluated the effects of regular bleach in BHI broth, demonstrated that while bleach inactivated *C. difficile* cells, there was no inactivation of spores (Edwards *et al.*, 2016). The authors conclude that the presence of organic material, which inactivates hypochlorite, reduced the effectiveness of sodium hypochlorite for spore inactivation.

4.6 Conclusions

The thermotolerance of *C. difficile* spores to the relevant laundry operating temperatures and the lack of sporicidal activity of both the reference detergent and the parts of the industrial detergents tested at operating temperatures, highlight the role of other factors in the laundry cycle, such as removal by agitation, surfactant action, other wash additives such as peracetic acid and the post-wash drying processes. Peracetic acid was not investigated in this study, but is included in the industrial wash cycle for neutralizing the bleach and for antimicrobial purposes. Therefore, the contribution of peracetic acid to the overall sporicidal effect of the industrial detergent system should be a focus for future study. Although organic matter has been shown to inactivate peracetic

acid *in vitro* and reduce sporicidal activity, so the protective effect of soiling, should also be considered.

The use of thermal disinfection alone, has been shown to require high temperatures for long exposure times. There are global drivers to reduce cost, energy and water use and deliver clean microbiologically safe linen. Therefore, the focus may move to the role of agitation, surfactant action and sporicidal compounds, with the aim of removing/inactivating pathogenic spores, at lower temperatures and for shorter exposure times. One such step could be understanding how to remove spores from linen and might start with exploring the nature of the adherence of spores to cotton fabric (section 5.2). This could be followed up with the testing of various detergents and additive compounds, to see if and how they affect the adherence of spores to cotton.

5 The *C. difficile* spore exosporium and adherence to cotton

5.1 Chapter abstract

Adherence of spores to linen may be a factor influencing *C. difficile* spore survival through a healthcare wash cycle. The spore exosporium may play a role in adherence to surfaces, such as stainless-steel, but spore adherence to cotton has not been investigated. The aim of this study was to quantify the adherence of *C. difficile* spores to cotton swatches over time. Adherence was quantified by inoculating spores onto swatches and removing by agitation. The proportion not recovered was determined to be adhered. The exosporium was partially or fully removed using trypsin and proteinase k, respectively and adherence was quantified. Removal of the exosporium was inferred by the corresponding reduction in hydrophobicity, by MATH assay. Spores were then imaged by SEM after 0 hours or 24 hours contact with cotton. The results showed *C. difficile* spores adhered to cotton over time; after 0 hours contact time there was 0% adherence of spores. Spore adherence increased to 51% after 24 hours contact time with cotton. Full removal of the exosporium, by proteinase k, significantly reduced adherence to 34% after 24 hours. SEM images of spores in contact with cotton for 24 hours, demonstrate the development of possible anchors, which may account for the adherence over time and influence survival through the healthcare wash by resisting removal. The length of time soiled linen is stored before laundering could impact the effectiveness of a healthcare compliant cycle and put patients at risk.

5.2 Introduction

Bacterial contamination of textiles and non-porous hard surfaces is a well-established problem in healthcare settings (Alfa *et al.*, 2008; Burden *et al.*, 2013; Zarpellon *et al.*, 2015; MacDonald *et al.*, 2016). There are several factors influencing the adherence of cells and spores to inorganic and organic surfaces, such as hydrophobicity of the cell, spore and surface, surface roughness and the exosporial layer (Faille *et al.*, 2010; Leishman *et al.*, 2010; Joshi *et al.*, 2012; Pizarro-Guajardo *et al.*, 2014; Díaz-González *et al.*, 2015).

Adherence to a surface begins with an initial phase where a spore comes in to contact with the surface through hydrophobic interactions, Van der Waals attraction or simple gravitational force (Gottenbos *et al.*, 2002). Husmark and Ronner (1990) demonstrated that *Bacillus* spp. spores were drawn to hydrophobic surfaces in the presence of hydrophilic media, but not in hydrophobic media e.g. ethanol. The physical roughness of the material can also influence bacterial adherence to and removal from some surfaces (Faille *et al.*, 2002). Roughness increases the physical surface area for bacterial adherence and may also protect cells from shearing forces (Katsikogianni & Missirlis, 2004; Wang *et al.*, 2009; Al Groosh, *et al.*, 2015). Aykent *et al.* (2000) demonstrated a positive correlation between surface roughness and *Streptococcus mutans* cell adhesion to dental implants. Furthermore, Wang *et al.* (2009) reported that a positive linear relationship between surface roughness values and residual

bacterial cell populations, after washing treatments with peroxyacetic acid, electrolysed water and SDW. In addition, topography of the surface material may play a part, where cells or spores may also be caught within micro-irregularities within the material (Flint *et al.*, 2000).

Conversely, Fernandes *et al.* (2014) demonstrated that roughness and hydrophobicity of organic surfaces, such as fruit, did not affect removal of pathogenic bacterial cells. This was supported by Jullien *et al.* (2003), who demonstrated that the roughness of inorganic surfaces such as stainless-steel, did not affect bacterial cell adherence.

Recently, surface roughness has been shown to effect cleaning efficacy in the presence of soiling, when attempting to remove spores from a surface. Gonzalez *et al.* (2017) demonstrated that roughened plastic coupons retained more simulated soil (blood) and more *Bacillus atropheus* spores, compared with smooth plastic coupons. When only spores were dried on the surface, the roughness of the coupon did not influence the ability of the spores to adhere to the surface. This suggests spores may adhere better to a rough surface, especially where soil is present. Furthermore, the cleaning agent used to remove the spores made no significant difference, where gauze soaked in sterile water, 70% ethanol or 0.5% sodium hypochlorite was able to remove similar levels of soil and spores.

With regard to cotton specifically, it has been shown that 100% cotton fibres have a relatively low bacterial cell-binding ability (Takashima *et al.*, 2004). In a study by Takashima *et al.* (2004) several strains of bacteria were compared, for 5/5 strains of MSSA the binding ratio was similar across strains for cotton, polyester and nylon. For wool, there was more variability across the strains (34.2% to 93.3%). Cotton (2%) and nylon (0.9%) had the lowest binding properties, whereas polyester had the highest at (96.2%). Furthermore, the authors suggest that ionic interactions may be involved, as sodium chloride solution at neutral pH caused partial inhibition of the binding of bacterial cells. Unfortunately, developing the concept of the cloth-binding property, to assess woven fabric rather than fibres, was not possible with the method employed by Takishima *et al.* (2004). This was because the test solution was absorbed by the fabric, which artificially increased the concentration of bacteria in the test solution.

Conversely, Bajpai *et al.* (2011) determined that *E. coli* cells adhered more effectively to 100% cotton woven fabric than to polyester woven fabric; presenting as single cells, paired cells and clusters on fabric surfaces, when viewed by SEM. Additionally, it was reported that as the contact time between the bacterial cells and fabric increased, so did the bacterial adherence. There was rapid adherence on 100% cotton between 5 and 10 hours contact time, which plateaued between 10 and 24 hours and the maximum adherence of the

E. coli was 0.71 mg/g (mg of cells/g of fabric) by 24 hours. In contrast, a much lower weight of cells was reported to adhere to 100% polyester, which increased linearly before reaching the maximum 0.34 mg/g by 24 hours.

There are no published studies investigating adherence of *C. difficile* spores to textiles, cotton fibres or woven fabric. Therefore to understand how adherence might occur, studies involving other surfaces were considered such as agar, glass stainless-steel and human cells (Panessa-Warren *et al.*, 1997; Paredes-Sabja and Sarker, 2012; Joshi *et al.*, 2012; Escobar-Cortés *et al.*, 2013). Panessa-Warren *et al.* (1997) demonstrated a number of small ‘bumps’ developed over the exosporium of *C. difficile* spores giving the spores a rough or ‘bumpy’ texture (Figure 5.4). It was seen that a tail or anchor developed at one end, which aided in attachment to agar, and co-agglutination with nearby spores as germination was initiated. Furthermore, Panessa-Warren *et al.* (1997) reports that after the thickened anchor had developed the spores were subjected to washing, agitation in sterile water at 300rpm, solvent baths, critical point drying and the spores were not dislodged. Interestingly, with the addition of EDTA, water and agitation, the spores were readily removed. This suggests that spores may form a reversible attachment, with others spores and/or the substrate they are present on and that this may be achieved with the development of a textured surface of the exosporium. The authors concluded that despite being ‘metabolically inactive’, *C. difficile* and *C. sporogenes* spores

were able to adhere well to agar before germination (Panessa-Warren *et al.*, 1997). The nature of spore adherence to surfaces was investigated further with developments in electron microscopy; Panessa-Warren *et al.* (2007) demonstrated activation and attachment of *C. difficile* (ATCC 9689 and 43594), *C. sporogenes* and *Bacillus* spp. spores to glass slides incubated aerobically at 100°C for 5 and 30 minutes; unexpectedly, this showed that attachment and the preliminary stage of germination was successful in air, without special growth conditions or nutrients in all species tested. The number of *C. difficile* (ATCC 9689 and 43594) spores attached to the slide after washing, was as high as the unwashed control counts. The authors note that moist-heat at 100°C did not kill the spores, allowing attachment and encouraging spore co-aggregation, using adjacent spores or organic debris as hold fasts. Furthermore, the attachment to the glass surface was sufficiently strong to withstand washing for 30 seconds in water and the subsequent washes required during Gram and Schaeffer-Fulton endospore staining. The authors suggested that the strong attachments seen under the study conditions were perhaps a type of pre-programmed response, independent of the usual requirements for *C. difficile* spore germination i.e. co-germinants and an anaerobic environment. It was also concluded that initial adherence was achieved by the 'bumpy' texture and then by development of the tail-like anchor; the point at which adherence had occurred and germination

was halted until the usual requirements were met, was not investigated and remains unclear.

The exosporium is the outermost layer of the spores of several bacterial species; it has been extensively studied in *B. anthracis* and *B. cereus* (Henriques and Moran, 2007; Barra-Carrasco *et al.*, 2013; Pizarro-Guajardo *et al.*, 2014; Díaz-González *et al.*, 2015; Li *et al.*, 2016). In *Bacillus* spp. the exosporium is a loosely-fitted outer layer composed of a smooth basal layer and a hair-like nap which plays a relevant role during early interactions with host cell surfaces (Xue *et al.*, 2011; Pizarro-Guajardo *et al.*, 2014). The hair-like nap layer is comprised mainly of BclA, a collagen-like protein and has been implicated in the adherence of *B. cereus* spores to inert surfaces in the food processing environment (Faille *et al.*, 2010; Lequette *et al.*, 2011). *B. anthracis* has been shown to have abundant BclA proteins within the exosporium, which facilitate the adherence of C3 complement protein enabling phagocytosis and dissemination within a host via macrophages (Bozue *et al.*, 2007).

Faille *et al.* (2002) found similar patterns of spore adherence to inert surfaces, such as stainless-steel, glass and Teflon, for spores of *B. cereus* and *B. subtilis*, despite their very different spore surface properties i.e. hydrophobicity, presence or absence of exosporium. The complex nature of adherence of spores to organic and inert surfaces, where hydrophobicity and elements of the

exosporium may play a role, suggests further work is needed to understand these interactions.

Most *C. difficile* strains produce spores with an exosporium that is tightly bound to the spore coat, where the exosporium has a hair-like nap layer similar to *Bacillus* spp. and a rough textured appearance (Panessa-Warren *et al.*, 1997; Panessa-Warren *et al.*, 2007; Díaz-González *et al.*, 2015). Spores of *C. difficile* 630 have a smoother exosporium, which is also tightly attached to the spore coat (Díaz-González *et al.*, 2015). Abhyankar *et al.* (2013) report finding the collagen-like protein BclA1, within the *C. difficile* exosporium, with the suggestion that it may be involved in adherence to surfaces. However, this conclusion appears to be based on the function of homologues within *Bacillus* spp. Furthermore, there were many uncharacterized exosporium proteins, found within the exosporium of *C. difficile*. These proteins may have unrecognized roles in adhering to surfaces, mainly because they have no known homologues in other better characterized species.

Pizarro-Guajardo *et al.* (2014) attempted to characterize the BclA1 protein within the exosporium of *C. difficile* spores. When the spores were treated with collagenase for 90 minutes to partially digest collagen-like protein BclA1, the spores retained their hydrophobicity to levels similar to untreated spores, but exhibited increased adherence to caco-2 cells. In *B. anthracis*, the

BclA proteins (BclA1 – BclA3) are the major contributors to spore hydrophobicity, suggesting that the BclAs of *C. difficile* and *B. anthracis* may have different functions. Lequette *et al.* (2011), found that adherence of *B. cereus* spores to stainless-steel was reduced in BclA⁻ mutant strains, which were unable to produce the protein, and there was a reduced spore hydrophobicity. The BclA collagen-like proteins may be involved in adherence of spores to inert surfaces, but not caco-2 cells or may have different properties in different species of spore-forming bacteria.

There is evidence that elements of the exosporium layer play a role in *C. difficile* spore adherence; removal of the exosporium, by sonication was shown to result in *C. difficile* spores with a significantly lower hydrophobicity (~20% vs ~60% for untreated spores) and significantly reduced adherence to caco-2 cells (Paredes-Sabja and Sarker, 2012). The correlation between exosporium removal and reduced hydrophobicity was supported by Escobar-Cortes *et al.* (2013), where proteinase k and trypsin were used to remove the exosporium, before conducting a hydrophobicity assay. The study demonstrated reduced hydrophobicity in treated spores, which was associated with either full or partial removal of the exosporium and confirmed by transmission electron microscopy (TEM) imaging. Furthermore, Joshi *et al.* (2012) demonstrated a link between *C. difficile* spore strain hydrophobicity and variable ability to adhere to stainless-steel. There was a large range in terms of proportion of spores binding to

stainless-steel, 14%-80% of spores were not recovered from inoculated steel coupons dependent on strain. Imaging of the steel coupons was not performed which may have provided qualitative assessment of the nature of the adherence, appearance of the exosporium and the activation status of the spores adhered.

The proteome of the *C. difficile* spore contains over 300 proteins; there are no known homologs or predicted function for over half of the proteins, but they are abundantly present within the spore and the exosporium (Lawley *et al.*, 2009; Abhyankar *et al.*, 2013; Díaz-González *et al.*, 2015). Furthermore, there could be selective advantages to adhering to inert surfaces in some species, depending on their aetiology. For example, *C. difficile* spore adherence to surfaces in the healthcare environment such as stainless-steel, cotton, polyester and plastics, or *B. anthracis* spore adherence to soil (earth) with more organic matter, where animals are more likely to be present (Williams *et al.*, 2013). There is much that could be learned with the continued characterisation of the *C. difficile* spore exosporium, spore-specific proteins and the nature of spore adherence to inert and organic surfaces.

Further work is needed to understand the role played by the *C. difficile* exosporium in adherence to surfaces and to aid decontamination of clinically relevant surfaces such as stainless-steel and cotton textiles including bed linen

and uniforms. Inhibition of adherence could be an effective way of enhancing the cleaning of textiles and hard surfaces, where detergents can be designed to remove spores more effectively. This could reduce the use of hazardous sporicidal agents such as sodium hypochlorite or hydrogen peroxide.

5.2.1 In summary

There are various mechanisms thought to play a part in the adherence of *C. difficile* spores to environmental surfaces. For example, hydrophobic and molecular interactions between elements of the exosporium layer of spores and inert surfaces, *C. difficile* spore activation and adherence can occur in aerobic conditions without nutrients or germinants present i.e. on a glass slide in room air at 100°C. There are no published studies exploring the adherence of *C. difficile* spores to cotton fabric over time. The present study focused on quantifying the adherence of spores to NHS (100% cotton) sheet material over time and investigating the role of the exosporium in adherence to NHS (100% cotton) sheet material.

5.2.2 Scope of the work

5.2.2.1 Aim

The aim of this investigation was to establish the role of the exosporium in the adherence of *C. difficile* spores to cotton.

5.2.2.2 Objectives

1. To quantify the adherence of *C. difficile* spores to NHS (100%) cotton sheet material immediately after inoculation and after 24 hours air drying.
2. To compare the adherence of *C. difficile* spores to NHS (100%) cotton sheet material immediately after inoculation and after 24 hours air drying, with either partial or total removal of the exosporium layer.
3. To determine changes in hydrophobicity of *C. difficile* spores after trypsin treatment (partial removal of exosporium) or proteinase k treatment (full removal of exosporium).
4. To visualize *C. difficile* spores on NHS (100% cotton) sheets after 0 hours and 24 hours contact time.

5.3 Methods

5.3.1 Generating spore suspensions for removal of exosporium

Type strain *C. difficile* NCTC 11209 spore suspensions were generated as previously described, using the long incubation (14 days in CMB) method (section 2.2.5.3). Spores were then recovered and purified in Histodenz density gradient medium as per Parades-sabja *et al.* (2012) (D1258, Sigma, UK). The Histodenz was made up at 50% w/v with SDW. Spore suspensions were centrifuged at 3000 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was re-suspended and washed with ice-cold SDW 10 times, with the final wash the pellet was re-suspended in 10ml SDW and layered onto 5ml of 50% Histodenz. The free spore phase was removed from the bottom of the layered suspension and resuspended in 10ml SDW.

5.3.2 Removal of exosporium

C. difficile spores NCTC 11209 ($7 \log_{10}$ cfu/ml) were incubated in either 25mM phosphate buffer alone (exosporium intact), phosphate buffer with trypsin (0.15 mg/ml) (T-1426, SIGMA, UK) (partial removal of exosporium), or proteinase k (0.3 mg/ml) with SDS (10 mg/ml) in phosphate buffer (P5568, SIGMA, UK) (complete removal of exosporium). The phosphate buffer was made up as previously described (section 3.2.5) and diluted with SDW to 25mM prior to use. The exosporium removal protocol was based on the method of Escobar-Cortes

et al. (2013). In brief, the spores were pelleted and re-suspended in 30 µl of the treatment solution, then incubated at 37°C for two hours with agitation at 200 rpm. The spores were washed five times in SDW, followed by enumeration to check viability. Treated spore suspensions were stored at -80°C until use. Hydrophobicity was also checked after treatment to infer the efficacy of the intended exosporium removal, which is thought to be correlated with a reduction in RH compared to un-treated spores (section 5.2.3).

5.3.3 Microbial adhesion to hydrocarbon test of hydrophobicity

The hydrophobicity of the spores of *C. difficile* strains from ribotypes 005, 002, 014, 11209 and 001/072 were assessed using the Microbial Adhesion to Hydrocarbon (MATH) test, as described in Joshi *et al.* (2012). All spore suspensions were generated and recovered as previously described (section 2.2.5.3). Trypsin, phosphate buffer and proteinase k treated spore suspensions were also analysed, they were generated and treated as previously described (section 5.2.2) Prior to testing all spore suspensions were centrifuged at 3000rpm for 1 minute and resuspended in SDW. Dilutions were made in SDW. Absorbance was measured using a spectrophotometer (Helios, Thermo, UK), where the absorbance of each spore suspension was measured at OD₆₀₀, and adjusted by dilution to between 0.5 and 0.6. Hexadecane was added to the spore suspension in a ratio of 4:1, where 0.5ml hexadecane was added to 2ml spore suspension. The test mixture was vortexed for two minutes and then

incubated at room temperature for 15 minutes. A 1ml sample of the aqueous layer was taken by pipette and the absorbance was measured. The hydrophobicity of each suspension was calculated as a percentage difference in absorbance before and after incubation in the presence of the hexadecane, using the formula: $100 - [(Final\ OD_{600} / initial\ OD_{600}) \times 100]$.

5.3.4 Quantifying the adherence of *C. difficile* spores to cotton after treatment to remove exosporium in part or in full

Cotton swatches (25cm²) were inoculated with 0.1ml of *C. difficile* NCTC 11209 spore suspensions treated with phosphate buffer, trypsin or proteinase K (5.0-5.5 log₍₁₀₎ cfu/ml) (section 5.2.2). The spores were recovered immediately, or after 24 hours air-drying, into 30ml MRD using the vortexing method (section 3.2.2 B). The recovered spore suspensions were serially diluted in MRD, duplicate 0.1ml samples were spread-plated onto BHIS/T and incubated anaerobically for 48 hours at 37°C. Calculations were carried out to assess the difference in relative adherence to the cotton fabric; the formula followed was: $100 - [(Final\ cfu/ml / Inoculated\ cfu/ml) \times 100]$.

5.3.5 SEM of *C. difficile* spores on NHS (100% cotton) sheet swatches

The SEM preparation and viewing were performed as previously described (Section 3.2.5). NHS (100% cotton) sheet swatches (25cm²) were inoculated

with 0.1ml of either a *C. difficile* NCTC 11209 or ribotype 001/072 spore suspension ($7 \log_{(10)} \text{cfu/ml}$). The swatches were then fixed immediately or after 24 hours drying at room temperature. In addition, a further set of swatches were inoculated with 0.1ml of a spore suspension treated with either phosphate buffer, trypsin or proteinase K (section 5.2.2).

5.3.6 Statistical analysis

The statistical analyses comprised Shapiro-Wilk normality tests to assess normal distribution, where the assumption of normality was confirmed independent t tests were performed comparing recovery at 0 hour and recovery at 24 hour for each treatment individually. Homogeneity of variance was compared using a Levene's test. Where the assumption of normality was violated, the Mann-Whitney *U* test was performed. The treatments from method 5.2.4 were analysed by ANOVA to compare each treatment at 0 hour air drying and 24 hour air drying. The significance level was set at $p \leq 0.05$. All investigations were carried out in triplicate on two separate occasions.

5.4 Results

5.4.1 Quantifying the adherence of *C. difficile* spores to NHS

(100% cotton) sheet swatches

There was a significant difference in the number of spores recovered from NHS (100% cotton) sheet swatches between 0 and 24 hours drying time, for buffer treated spores (4.02 vs 3.78 log₍₁₀₎ cfu/ml, $t=2.475$, $p\leq 0.05$) and trypsin treated spores (4.32 vs 4.04 log₍₁₀₎ cfu/ml, $t=2.950$, $p\leq 0.05$). There was no significant difference between the recovery of spores at 0 and 24 hours when treated with proteinase K (3.65 vs 3.58 log₍₁₀₎ cfu/ml, $U=32.000$, $p>0.05$) (Figure 5.1).

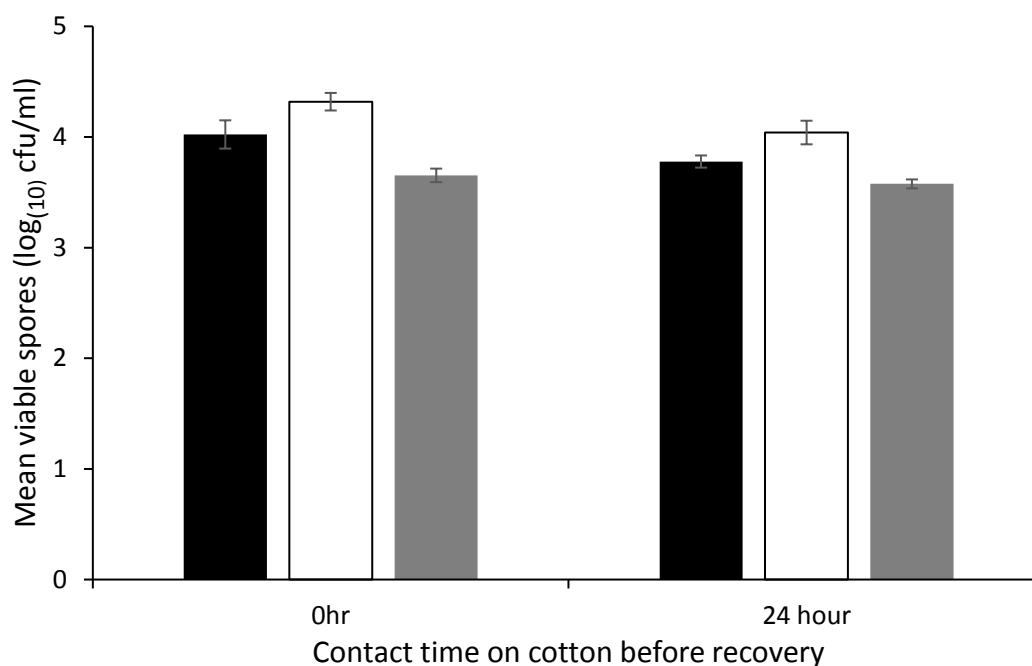


Figure 5.1. *C. difficile* spores recovered from NHS (100% cotton) swatches after 0 or 24 hour air-drying. Spores suspensions were treated with buffer (■), trypsin (□) or proteinase K (■) (mean \pm SE, $n=6$).

The relative adherence of spores for each of the treatments was calculated, as was the RH of treated spores (Table 5.1). The findings showed that the buffer treated spore suspension had no adherence at 0 hour, with recovery of almost 100% of the spores inoculated when recovered immediately (4.02 of 4.07 log₍₁₀₎ cfu/ml recovered). This increased to 51.78% adherence when spores were recovered from the swatches after 24 hours drying time (3.78 of 4.07 log₍₁₀₎ cfu/ml recovered).

Table 5.1. Relative adherence of *C. difficile* spores (11209) to NHS (100% cotton) sheet swatches after 0 hours or 24 hours drying time and RH by MATH assay (% decrease in OD₆₀₀ after exposure to Hexadecane). Spores were previously treated with buffer (exosporium intact), trypsin (partial removal of exosporium) or proteinase k (full removal of exosporium) (mean, *n*=6).

Treatment	0 hour (%)	24 hour (%)	Hydrophobicity (%)
Buffer	0	51.78	63.2
Trypsin	12.88	51.82	34.7
Proteinase K	19.76	34.79	4.1

With the trypsin treated spores there was 12.88% adherence when spores were recovered immediately (4.32 of 4.53 log₍₁₀₎ cfu/ml recovered) and 51.82% adherence after 24 hours drying time (4.04 of 4.53 log₍₁₀₎ cfu/ml recovered). When the spores had been treated with proteinase K, for full removal of the

exosporium, there was 19.76% adherence when the spores were recovered immediately (3.65 of 4.13 log₍₁₀₎ cfu/ml recovered), compared to 34.79% after 24 hours air-drying (3.58 of 4.13 log₍₁₀₎ cfu/ml recovered).

The RH score for buffer treated spores was 63.2% in contrast to trypsin and proteinase k which were 34.7% and 4.1%, respectively. The descending hydrophobicity scores of spore suspensions treated with trypsin and proteinase k, suggests that the exosporium had been either partially (trypsin) or completely removed (proteinase k) (Table 5.1).

5.4.2 Microbial adhesion to hydrocarbon test of hydrophobicity

The spores of *C. difficile* NCTC 11209, had a significantly higher RH compared to all other clinical ribotypes tested ($F=7.726$, $p\leq 0.05$). The RH of all other ribotypes was not significantly different ($p>0.05$) (Figure 5.2).

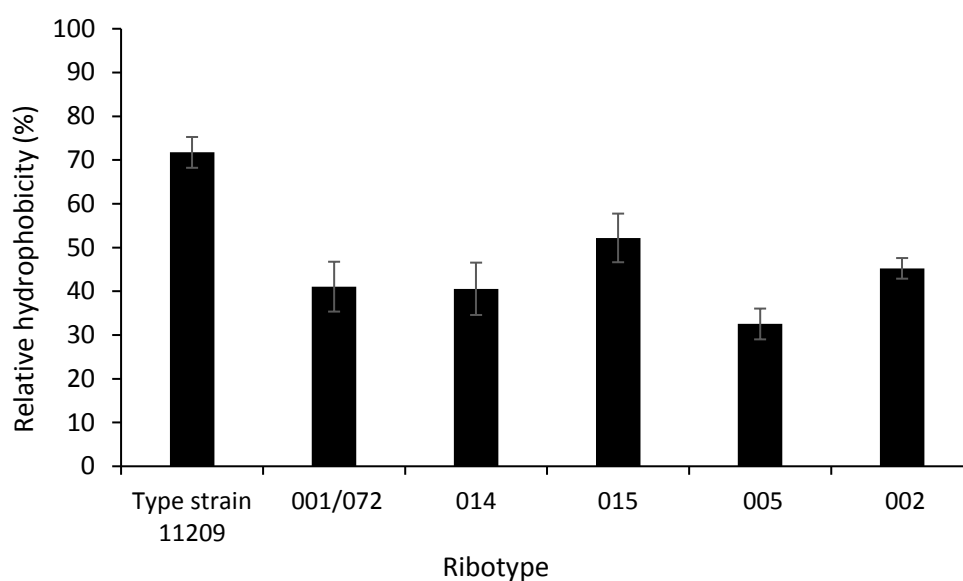


Figure 5.2. Relative hydrophobicity of *C. difficile* spore suspensions from clinically relevant ribotypes (from PHE); NCTC 11209 and field recovered strain ribotype 001/072 (Mean, \pm SE, $n=6$).

5.4.3 SEM of *C. difficile* spores on NHS (100% cotton) sheet

swatches

In the SEM images it was shown how a ribotype 001/072 spore adhered to the surface of the cotton fibre, with a possible anchor region (Figure 5.3 D). This was evident on the swatch fixed after 24 hours air drying, but not on spores fixed immediately, for the spores of ribotype 001/072. The spore shown for NCTC 11209 at 24 hours, could have a possible anchor, but it isn't as clear (Figure 5.3 B). The spores in all images exhibited a rough bumpy texture, although those of NCTC 11209 looked more uniform and those of 001/072 had a more irregular

pattern. Images of spores fixed immediately, show shorter more ovoid shaped spores (Figure 5.3 A and C).

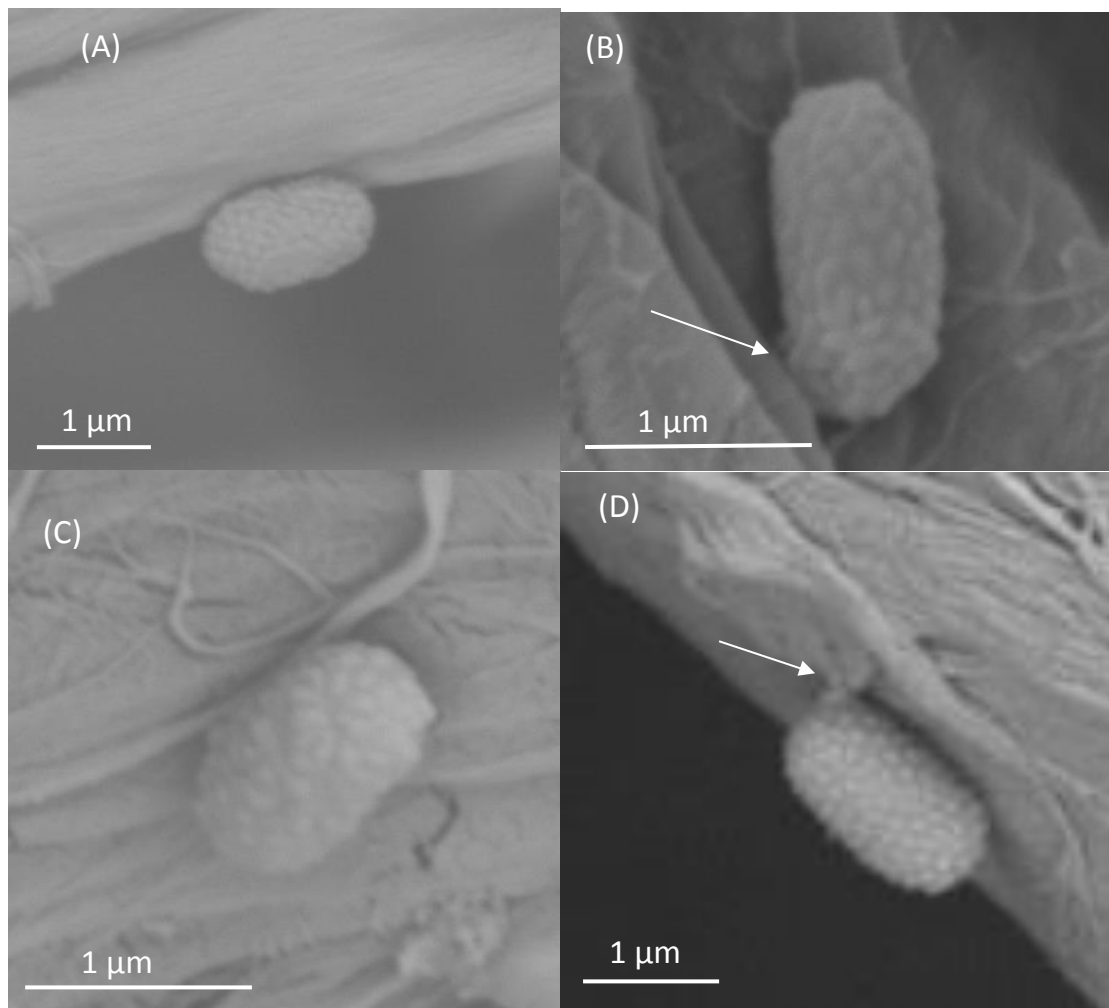


Figure 5.3. *C. difficile* spores on 100% cotton swatches (A) *C. difficile* NCTC 11209 spore fixed immediately after inoculation, (B) *C. difficile* NCTC 11209 spore fixed after 24 hours drying time (white arrow = possible anchor structure), (C) *C. difficile* ribotype 001/072 spores fixed immediately after inoculation and (D) *C. difficile* ribotype 001/072 spores fixed after 24 hours air-drying. (White arrow = possible anchor structure).

5.5 Discussion

5.5.1 Quantifying the adherence of *C. difficile* spores to NHS (100% cotton) sheet swatches

The rate of adherence of *C. difficile* spores to NHS (100% cotton) sheet swatches was quantified immediately after inoculation and after 24 hours air-drying. It was seen that the spores which were air-dried were able to remain adhered to the cotton after vortexing, suggesting there was a strong attachment between spore and fibres. The significant difference in number of spores recovered when swatches were vortexed immediately vs air drying for 24 hours, suggests that over the drying time, half of the spores were able to adhere to the fabric (Figure 5.1). This suggests that delays in laundering cotton textiles, could allow spores time to adhere in a way that prevents removal during laundering. This is in agreement with Panessa-Warren *et al.* (1997), where *C. difficile* spores were found to attach to BHI agar within 2 hours of anaerobic incubation and resisted removal by agitation for 30 minutes at 300 rpm. To briefly reiterate, Panessa-Warren *et al.* (1997) found that adherence was facilitated by an anchor which developed when the spore began the preliminary stages of germination, in an aerobic environment (Figure 5.4). The high level of adherence of buffer treated spores after 24 hour drying time is in agreement with Bajpal *et al.* (2011), where *E. coli* cells were shown to adhere to 100% cotton fabric over time with increasing levels of adherence up to 24 hours; although longer times were not

assessed either in Bajpal *et al.* (2011) nor by the present study. The present study's findings of 50% adherence of spores after 24 hours is in contrast to Takishima *et al.* (2004), where MRSA/MSSA bacterial cells showed the low affinity (2%) for 100% cotton. In Takishima *et al.* (2004) the cells in suspension were incubated with the fibres at 18 rpm for only 1 hour before the number of remaining cells in suspension were quantified, so the reduced time the MRSA/MSSA cells were in contact with the fibres, may account for the difference in adherence to the present study. Alternatively, cell and spore adherence to cotton may be different, which could be due to properties of the cells or spores and the nature of the underlying mechanism of adherence e.g. spores may be 'pre-programmed' to adhere to a substrate prior to germination (Panessa-Warren *et al.*, 2007).

It could be hypothesised that adherence to a substrate could have a positive influence on germination. In early testing of taurocholate sensitivity, *C. difficile* spores germinated at 60% efficiency in broth containing taurocholate (10%) compared to BHI agar with taurocholate (0.1%) (Sorg and Sonenshein, 2008). Furthermore, rapid adherence to glass was demonstrated in a wide range of spore-forming bacteria such as *C. difficile*, *C. sporogenes*, *B. cereus*, *Bacillus licheniformis*, *Bacillus stearothermophilus* and *Bacillus pumilus* (Panessa-Warren *et al.*, 2007). These species represent pathogenic, environmental and

thermophile strains, which all had rapid and strong adherence to glass under extreme conditions (aerobic incubation for 5 or 30 minutes at 100°C).

The spore suspensions treated in trypsin and proteinase k solutions, showed a marked reduction in hydrophobicity compared to buffer treated spores in line with the findings of Parades-sabja *et al.* (2012) and Escobar-Cortes *et al.* (2013). As expected, the proteinase k treatment had the greatest reduction in hydrophobicity of spores, which is considered indicative of removal of the exosporium layer (Table 5.1). Trypsin treatment caused a subtler reduction in hydrophobicity; this was expected as trypsin has been shown to only partially remove the exosporium, when used independently of sonication (Paredes-Sabja and Sarker, 2012; Joshi *et al.*, 2012). These findings suggest that expected partial or full removal of the exosporium had taken place; the next step would be the definitive confirmation by SEM imaging either of treated spores on NHS (100% cotton) sheet swatches or treated spore samples independent of the swatches.

Adherence results were interpreted under the putative assumption that the exosporium had been either partially or fully removed by the treatments described. The spores treated with proteinase k had the highest adherence at 0 hour, with 34% adhered when spores were recovered immediately after inoculation (Table 5.1). In contrast, the proteinase k treated spores had a

significantly lower relative adherence, than buffer treated spores, when the swatches were air dried for 24 hours. The better adherence at 0 hour was not expected, it is not clear why this should be the case. The reduced adherence at 24 hours, compared to buffer treated spores, was expected as the exosporium was removed and elements which may mediate adherence were no longer present. In contrast, with trypsin treated spores, there was low level adherence at 0 hours, but after 24 hours air drying the adherence was the same as the buffer treated spores (around 51%). This pattern was also seen with adherence to human caco-2 cells, where full removal of the exosporium (by sonication followed by trypsin treatment) resulted in fewer spores adhering to the human epithelial cells (Paredes-Sabja and Sarker, 2012). It was reported by Paredes-Sabja and Sarker (2012) that after treatment by sonication (followed by 18 hour trypsin digest), two uncharacterized proteins were released which were thought to usually be present in the exosporium. The released proteins were thought to be important in adhering to the caco-2 cells, as their removal reduced adherence to caco-2 cells. Therefore, the authors concluded that the exosporium layer plays a role in adherence to cells *in vivo*.

In summary, the RH of the test strain NCTC 11209, was significantly different from all other clinical ribotypes tested. This may affect adherence properties as hydrophobicity is thought to play a role and may also make spores of NCTC 11209 able to adhere to surfaces particularly well. The reduction in RH

of the treated NCTC 11209 spore suspensions suggests that removal of the exosporium, either partially or fully, had taken place. Furthermore, the relative adherence of spores to cotton after 24 hours air drying, was reduced, when the exosporium was considered to be fully removed. These findings suggest the exosporium is important in adhering to NHS (100% cotton) sheets and that if spores are left to air dry on the sheets, before being laundered the spores may develop a strong adherence which may prevent removal by the agitation of a wash cycle.

5.5.2 *C. difficile* spores on cotton after 0 hour and 24 hours

The SEM images further supported the evidence of spores adhering to cotton over time. There was a possible anchor or connection between the spores and the cotton fibres after 24 hours air drying, rather than being caught in the weave of the fabric. This was further shown to involve the exosporium directly, where spores which had putatively had the exosporium removed (as discerned by reduction in hydrophobicity) were less able to adhere after 24 hours drying time. The adherence of spores may be further elucidated in several ways, TEM/SEM of the proteinase K treated spores could confirm removal of exosporium, which would show the exosporium is at least partly responsible for adherence. In addition, collagenase could be used to specifically degrade the BclA collagen-like proteins which are abundant on the exosporium and then adherence of the collagenase treated spores could be quantified again. This

could help identify the role, if any, of the BclA proteins in spore adherence to cotton.

5.5.3 SEM of *C. difficile* spores on NHS (100% cotton) sheet swatches

The present study is the first to show *C. difficile* spores may adhere to 100% cotton in an aerobic environment, exhibiting possible anchor structures (Figure 5.3 B and D), similar to what has been seen when spores adhere to BHI agar (Panessa-Warren *et al.*, 1997; Panessa-Warren *et al.*, 2007) (Figure 5.4 C). The anchor structure appears to extend from the exosporium to the cotton fibre (Figure 5.3 D). Dormant *C. difficile* spores have previously been shown to be small, elliptical and have a smooth appearance (Figure 5.4 A); activated spores, by contrast, appear elongated and have a rough texture with regular ‘bumps’ over the surface (Figure 5.4 B). Similarly, all the spores seen in the present study, had the rough texture with regular ‘bumps’ over the surface layer (Figure 5.3); this suggests the spores were not dormant, but activated and ready to germinate in the right conditions. The rough texture, which suggests activation, may be explained by the short storage time of spore suspensions, between inducing sporulation and their subsequent use in investigations. With a short storage time the spores may not have entered a dormant state and developed the smooth exosporium seen on dormant spores.

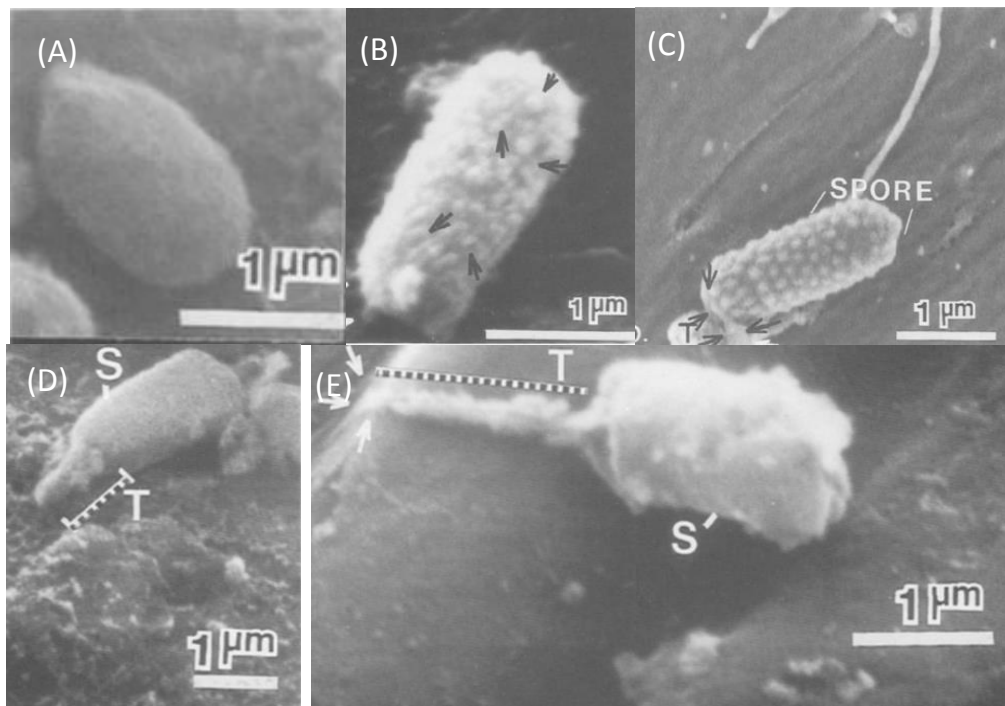


Figure 5.4. *C. difficile* spores (A) a dormant spore, (B) an activated spore with 'bumpy' exosporial surface, (C) a spore with thickened tail region adhered to agar in the preliminary stage of germination, (D) during germination the spore and tail become smooth, (E) late stage germination: after agitation with SDW the spore is still adhered (from Panessa-Warren *et al.*, 1997).

5.6 Conclusions

By understanding the process by which spores adhere to clinically relevant surfaces such as cotton, stainless-steel and plastics, strategies may be developed that inhibit attachment and aid decontamination processes. The present study provides further evidence that the *C. difficile* exosporium is important in adherence and contributes to the hydrophobicity of the spore surface. In addition, there was the first evidence of a role of the exosporium in the adherence of spores to cotton specifically.

The exact elements that contribute to the spore hydrophobicity are unknown, but are likely to reside in the outer exosporium as removal by trypsin and proteinase k, resulted in a reduced RH. Without the exosporium, the proteinase k treated spores had a reduced relative adherence to cotton after 24 hours air drying. Therefore, the exosporium or the hydrophobicity conferred by elements in the exosporium are likely to have influenced adherence to cotton. Trypsin was inferred to have caused a partial removal of the exosporium and was also able to reduce the spore RH; unexpectedly it had no effect on relative adherence, compared to buffer treated spores, when swatches were air dried for 24 hours. The increased adherence over time of intact spores to dry cotton, poses interesting opportunities for ensuring decontaminated textiles. If adherence could be inhibited, spores may be removed more easily and aid in providing safe decontaminated linen for vulnerable patients.

6 Discussion

6.1 Quantification of the survival of *C. difficile* spores on NHS (100% cotton) bed sheets after washing under chemo-thermal disinfection conditions which meet the NHS Policy HTM 01-04 (2016).

The aim of the study was to quantify survival of *C. difficile* spores on infected linen washed to the minimum requirements of the HTM 01-04 healthcare laundry policy. The policy had been updated throughout the time of the study, but the basic requirements for thermal disinfection have remained the same. Independent quantification was completed in two ways, a simulated healthcare laundry cycle and an *in situ* test at a commercial laundry. The simulation demonstrated that the policy compliant cycle, the industrial detergent and thermal disinfection were able to reduce the *C. difficile* spore contamination significantly, by $>6 \log_{(10)}$. However, a low level of spore contamination was still recovered ($0.1-1 \log_{(10)}$) (Table 3.2). Furthermore, it was seen that the detergent was only able to reduce spore load by $2.81 \log_{(10)}$ cfu/ml during *in vitro* tests of sporicidal activity (at 71°C), therefore factors in the wash cycle simulation, beyond inactivation by chemo-thermal means were responsible (Figure 4.4). The first factor, was likely to be surfactant action, whereby the detergent removed hydrophobic soil. However, significantly different relative hydrophobicity, did not affect the combined ability of the detergent and

agitation to remove the spores of NCTC 11209 (RH=71.74%) and field strain ribotype 001/072 (RH=41.06%) in the simulation (Figure 3.3 and Figure 5.2).

Furthermore, there may also be an affect at higher temperatures on the surfactant action. It was recently shown that the number *C. difficile* spores recovered from swatches was reduced by 1 log₍₁₀₎ when washed at 60°C (Mackay et al., 2017). Where 60°C was unable to inactivate the spores and the detergent was unable to reduce the contamination, either by sporicidal activity or surfactant action. The detergent used in Mackay *et al.* (2017) was different to the industrial detergent in the present study, therefore there may be differences in what may be seen with the industrial detergent used in the present study, should it be used at 60°C.

Conversely, Sooklal *et al.*, (2014) found that when bleach was accidentally omitted from a reusable mop head washer, the detergent, thermal disinfection and agitation combined were unable to decontaminate mop heads and hospital CDI rates increased. This not only illustrates the importance of the disinfection chemistry, and the use of bleach in CDI control, but also provides a possible link between spores surviving a laundering cycle, re-entry to a ward and consequently an increased infection rates. The CDI rate reduced to a normal level when the machine fault was corrected and bleach once more used in the

cycle (Sooklal *et al.*, 2014). This highlights the use of bleach for inactivating spores.

In the present study it was independently shown that the WE wash cycle at the commercial laundry failed to meet the microbiological standards required by HTM 01-04 (e.g. $>5 \log_{(10)}$ reduction, <100 cfu, no pathogenic bacteria). There were a small number of spores surviving the chemo-thermal conditions of a HTM 01-04 compliant WE cycle and *in vitro* tests of the thermal disinfection temperature. This may pose a significant risk if the spores return to hospital wards on processed linen; particularly if spore contaminated linen enters different wards without CDI patients where infection control, cleaning regimes or antibiotic prescribing may not take environmental spore contamination in to account.

The spores which had survived the infected linen wash and simulation may have remained on the linen because of an active adherence process, as seen in Panessa-Warren *et al.* (1997) and in the SEM images of the present study, where spores had developed a possible anchor after being air dried for 24 hours (Figure 5.3 D and Figure 5.4 B). When this was quantified as relative adherence of spores, there was a significant increase after 24 hours from 0% - 51% (Table 5.1). Considering the significant adherence of spores to cotton over time and the SEM images of adhered spores, it could be hypothesised that when

spores are shed onto bed sheets, they develop a strong adherence to the cotton fibres, when infected linen is stored before collection and may resist removal during the wash cycle.

It was demonstrated that vortexing was the most efficient method of recovering *C. difficile* spores from NHS (100% cotton) sheet material when the spores had been inoculated in the presence of soiling (Figure 3.2). While this was vital for performing the subsequent investigations, it also highlights the need for a defined method of agitation that may be used for validating the disinfection cycle across all healthcare laundry providers. For example, the stomaching method (1 minute) was used at the collaborating commercial laundry to perform internal quality control; this was shown to be inferior to the vortexing method (Figure 3.2). Adopting the vortexing method described in the present study could improve the quality control of laundered healthcare linen and make the linen quality assessment standardised across all validating laboratories.

There were differences between the proportion of spores recovered by vortexing, when spore suspensions had been purified, using Histodenz density gradient media and when suspensions were not purified. Recovering purified spores from NHS (100% cotton) sheets (section 5.3.1), facilitated the removal of a greater proportion of spores (49% vs 31% unpurified). One reason for this

could be the lysed cells and cell debris facilitating adherence over the air-drying period in the method comparisons made with unpurified spores (section 3.2.2). It has been shown that where soiling and other spores are present the adherence of spores can be increased and form co-aggregates (Panessa-Warren *et al.*, 1997; Panessa-Warren *et al.*, 2007; Gonzalez *et al.*, 2017).

In care facilities, where asymptomatic carriage of *C. difficile* is unrecognised there may be bed sheets being washed using the standard laundry process, instead of the enhanced process for infected linen. This could result in high levels of *C. difficile* spore contamination because the reference domestic detergent was shown to not have sporicidal activity at 71°C (Figure 4.3) and a recent study, by Mackay *et al.* (2017), demonstrated 1 log₍₁₀₎ reduction in spores after a 60°C wash simulation (leaving 5 log₍₁₀₎ cfu/swatch).

With an aging population there are likely to be many more susceptible hosts, a shift has been observed towards a large proportion (up to 46%) of new cases of CDI being found in long-term elderly care facilities (Kim *et al.*, 2011; Garg *et al.*, 2013). This is not surprising given the high-risk factors associated with the type of residents e.g. >65 years old, high proportion of co-morbidities and high levels of antibiotic usage. When this is coupled with high levels of asymptomatic carriage in care facilities, up to 58%, and the ability to shed large numbers of

spores (up to 5.9 log₍₁₀₎ cfu/g faeces) it may be beneficial to use an enhanced process routinely, particularly in long-term care facilities for elderly people.

6.2 Comparison of the effects of heat, detergent and soiling on the inactivation of *C. difficile* spores *in vitro*

The extreme thermotolerance of *C. difficile* spores at 71°C and 90°C demonstrates the need for additional measures for decontaminating infected linen covered by the HTM 01-04 laundry policy. Thermal disinfection alone, will not decontaminate spores, detergents improve the rate of disinfection by removing and inactivating spores, but the policy must go further in providing information on how to implement and provide a compulsory validation method for those alternative disinfection methods.

When a reference domestic detergent was investigated at room temperature and 71°C there was little sporicidal activity despite the presence of bleaching agents (peroxide) in the detergent. The sporicidal activity seen at 90°C, was due to entirely to the heat alone. Additional information about the domestic detergents performance at 60°C would be useful, due to the relevance to domestic machines and care homes which were running a standard wash. However, at 90°C the domestic detergent could reduce the spore load by 3 log₍₁₀₎ as long as the temperature was held for a minimum of 10 minutes (Figure 4.3). In care facilities which use a domestic washing machine and detergent

combination (routinely washing <71°C), spores may survive on linen. With those facilities washing at 71°C using an industrial machine and detergent there is a strong likelihood that should any *C. difficile* spores enter the wash cycle, there may be low level survival of spores on the linen.

For a full assessment of the decontamination effects of temperature and industrial detergent, without the surfactant actions and rinsing, a test protocol could be developed using a semi-permeable pouch which could hold spores; this is currently used in the laundry industry for vegetative cells only. Developing a semi-permeable pouch which can hold *C. difficile* spores would be an effective way of comparing the efficacy of industrial washes across providers where number and temperature of rinses, chemical disinfection and water use vary significantly. The current microbiological assessment methods cannot accurately compare efficacy of the variety of healthcare compliant washes. The current method is to sample a fully processed sheet on a regular basis to assess the overall quality of finished linen, but the precise method of agitation is merely suggested in HTM 01-04, not explicitly required. It is feasible that different validation protocols could give different results and in theory detergent companies validating their own disinfection cycles, could choose less vigorous methods of recovering spores and cells to suit their needs. There was a trend in the comparison of recovery methods for the stomaching method to recover a greater number of spores with longer times and with a higher setting; this was

significantly different when comparing 30 seconds/low to 1 minute/high (Figure 3.1). It is important that inter-laboratory variation is kept to a minimum, so that results can be compared, therefore it is unclear why a standard, effective method is not in place.

One final consideration, was the variation in the effectiveness of the simulation ($>6 \log_{(10)}$ reduction) and the *in situ* study of the commercial WE cycle ($<1 \log_{(10)}$ reduction) (Figure 3.3 and Table 3.2). This may be due to the presence of alginate bags in the commercial WE cycle and not in the simulated wash; once the alginate is in solution it has the potential to act as soiling and be a protective agent. Alginate is a polysaccharide commonly produced by seaweed, it is also produced by *P. aeruginosa*, in the production of biofilms which can provide protection from antibiotics (Hentzer *et al.*, 2001; Ghadaksaz *et al.*, 2015). The alginate in solution may be preventing contact with the detergent, and thereby reducing the removal and any sporicidal activity of the chemical additives.

6.3 Establish the role of the exosporium in the adherence of *C. difficile* spores to cotton

There were two relevant findings in the adherence investigation, first was the increase in adherence of spores to cotton over 24 hours and second was the

role of the exosporium in that adherence. Where the inferred removal of the *C. difficile* exosporium, reduced the adherence of spores to cotton.

With the evidence of increasing spore adherence to cotton over time, it is important to note that the spores on the experimental (100% cotton) sheets in the commercial laundry investigation had been air-dried for ~48 hours in storage before collection (section 3.2.6.3). The prolonged storage of infected linen may have contributed to the strong adherence of spores to the linen and the recovery of viable spores after a high temperature wash (Table 3.2). Understanding the relationship between adherence and contact time could be used to suggest shorter storage times and may impact the level of spores able to adhere through the wash. This could mean more regular collection of infected linen, washed promptly and not stored, this may lead to the linen may be more thoroughly decontaminated.

Furthermore, with the evidence of spores adhering over time, this may suggest sampling techniques which do not use sufficient agitation, i.e. contact plates or slit air samplers, may be underestimating the number of spores on a surface or textile item. For example, using a slit sampler in Perry *et al.* (2001), 1-10 cfu of *C. difficile* spores were found on 7 uniforms, whereas samples of non-spore-forming species were found at levels of >100 cfu per uniform.

It could be hypothesised that there may be a selective advantage with the ability to adhere to inert surfaces. For example, cotton is a polymer comprising 90% cellulose, which is found in plant cell walls and is produced by a number of bacterial species. Adhering to plants may increase the spores' likelihood of being ingested, particularly for species which are adapted for conditions in the mammalian gut, such as *C. difficile* (Williams *et al.*, 2013).

However, for this to have an influence on CDI rates, there must be a reversible nature to the adherence, especially as the vegetative cell is anaerobic and the spore must enter a host before germination. The next step in understanding spore adherence is yet to be explored, there may be a mechanism by which spores adhere to hard surfaces or textiles, but then disengage, perhaps when the spores encounter an organic surface. There is a suggestion of a reversible nature to the adherence of *C. difficile* spores to the surface of agar, where Panessa-Warren *et al.* (2007) demonstrated the complete disruption of spore adherence to agar by washing the spores in EDTA or barium once they had adhered under optimal conditions. The authors, proposed that cations, (Ca^{2+} and Mg^{2+}) may be needed for adherence. Therefore, neutralizing cations on contaminated surfaces or in the wash could provide a way of interfering with the spores' ability to adhere and facilitate removal from surfaces or complete decontamination of infected linen; whereby spores are rinsed away after 'disengaging' from the NHS (100% cotton) sheets.

The use of detergent builders, which hold free cations may be a potential research area for enhancing the removal of spores from textiles and hard surfaces.

6.4 In summary

It was shown that the suggested method of agitation, for microbiological assessment, is likely to be underestimating the number of *C. difficile* spores present on linen processed in a commercial or onsite laundry. Furthermore, linen processed in a WE on a HTM 01-04 compliant infected linen cycle may have a low level of viable spores which can be recovered. The main factors which enabled the reduction of $>6 \log_{(10)}$ spores in simulations were likely to be the surfactant action of the detergent and the agitation of the wash cycle; the industrial detergent components tested had limited sporicidal activity at 25°C and moderate activity at 71°C (Figure 4.4), where the detergent and high temperature worked synergistically to inactivate spores. With the added effect of spore removal from the swatches, there was a much greater reduction in recovered spores in the simulated wash (Figure 3.3). There were far fewer spores on the naturally contaminated sheets than previous estimates of shedding would suggest are likely (Table 3.2). However, the processed linen from the commercial laundry returned similar levels of residual surviving spores to the simulation investigations.

Despite the presence of low levels of spores which may remain on linen washed in a WE, the contribution of inadequately decontaminated linen to active CDIs is likely to be small, given the reduction in rates in recent years. The step between a low level of spores on processed linen entering a hospital and subsequent CDI is yet to be established. However, the infectious dose is presumed to be very small, so the possibility cannot be discounted that spores from inadequately laundered linen may be causing infections. It is also possible that spores on inadequately laundered linen could cause asymptomatic carriage, which may be hidden until later antibiotic usage. The background rate of *C. difficile* remains fairly constant despite cleaning improvements, better antibiotic stewardship, improved hand hygiene and training in barrier measures. It is important that future work continues to assess the likely sources leading to transmission of *C. difficile* and to work on improving the laundry policy to protect vulnerable older patients in hospital or long-term care who may be exposed though inadequately decontaminated linen.

6.5 Future studies

In order for the research conducted to have greater impact it will be important to continue the work with the commercial laundry industry to develop a way of quantifying spore levels before and after the entire healthcare wash process, including WE pre-wash and CTW main wash. Due to limitations in identifying and recapturing the linen at the facility used in this study, this was not possible.

It may be that the complete cycle (which processes millions of items a week), may be more effective than the WE cycle with drying and finishing. In which case, the focus can move to ensuring WE cycles, important for the decontamination of infected laundry at commercial facilities and onsite in care facilities, are improved.

With the drive to reduce energy and water usage the influence of each of the wash parameters on the decontamination of spores from linen could be further explored to enable more eco-friendly cycles to be developed which still deliver microbiologically safe linen. This could be achieved by investigating the outcomes for spores in the simulation, by sampling the waste water to quantify the concentration of viable spores leaving the machine. Spores could be sampled from waste water and swatches at different points in the wash cycle such as, after the first main wash cycle, after the bleach rinse and again after the peracetic acid rinse. This would give more detail as to whether spores were either inactivated, or alternatively removed with subsequent transfer to sterile swatches in varying proportions or removed in the rinse water.

The next step in linking surviving spores to CDI transmission is outside the scope of this work, but could be explored by using inoculated sheets for bed-making in an experimental hospital room. Samples from the air and the environment could be taken at various distances from the bed after bed-making

with contaminated sheets; this would assess how sheets contaminated with low levels of spores may contribute to the contamination of the near-patient environment.

Future work could also look at adherence over a longer time as the sheets in hospitals are likely to be stored for much longer than 24 hours drying time and could be one reason why there was similar levels of spores recovered after the WE cycle *in situ* compared to the optimised simulation, despite the different original spore loads. If the time air drying was increased to 48 or 60 hours it may be that a greater proportion of the spores may adhere well, survive the simulation and be recovered.

7 Recommendations

The following are recommendations for improving the decontamination of infected linen contaminated with *C. difficile* spores, based on the findings of this investigation:

1. When providing quality assurance of processed linen, all laundry operators must ensure they use a standard method which removes the maximum number of spores and allows comparison across sites i.e. vortexing is preferable to stomaching.
2. Public reporting of the results of the quality assurance tests performed; WE cycles were independently demonstrated (by simulation and *in situ* at the commercial laundry) to be unable to meet microbiological test standards e.g. $>5 \log_{(10)}$ reduction, <100 cfu and no pathogenic bacteria.
3. The design and implementation of a semi-permeable dose pouch containing *C. difficile* spores (and/or *B. cereus* spores) to test for sporicidal activity of the entire cycle, as part of an annual validation and if any changes are made to the cycle e.g. change in temperature or change of detergent.
4. The development of detergents which can inactivate or remove the remaining spores recovered after the washing, drying and finishing cycles in a WE cycle. The focus could be to inhibit adherence (by neutralising

cations) or have added capacity to inactivate spores, especially if reducing temperatures and/or water usage.

5. Infected linen should be washed as soon as possible after removal from beds to prevent spores from developing a strong adherence to the cotton fibres, allowing passage through a WE cycle including washing, drying and finishing.
6. The two step wash protocol with WE pre-wash and CTW main wash still needs to be validated independently, to confirm it performs better than the WE cycle alone.

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Appendix 1 - Long-term care facility questionnaire

SECTION 1 – BACKGROND INFORMATION

1) Location of facility – county:

2) Does the facility care for clients with any of the following (tick all that apply):

- ☐ MRSA
- ☐ C.diff / C. difficile
- ☐ Fungal infections
- ☐ Other – please specify

3) What material/s are the facility's bed sheets made from (tick one):

- ☐ 100% Cotton
- ☐ Polycotton blend
- ☐ Other – please specify

4) How are soiled bed sheets washed at the care facility (tick one)?

- ☐ On site laundry - (please go to **Section 2**)
- ☐ By a commercial laundry facility - (please go to **Section 3**)

SECTION 2 – ON SITE LAUNDRY INFORMATION

- 1) Which make and model of washing machine do you use?

- 2) Which brand of detergent or detergent system, if any, do you use?

- 3) What temperature washing cycle do you use for soiled bed sheets? e.g. maximum temperature reached and how many minutes the temperature is held

- 4) How long is the whole cycle for washing soiled bed sheets?

- 5) For soiled bed sheets do you use (please tick all that apply):

- ☐ Pre-wash cycle
☐ Sluice process
☐ Soluble bags
☐ Neither / Unknown

- 6) How do you dry/finish sheets after they have been washed?

- 7) Which company verified the laundry process at the facility? Do they do an annual validation of the decontamination cycle?

THANK YOU FOR YOUR TIME

SECTION 3 – COMMERCIAL LAUNDRY INFORMATION

1) Which commercial laundry company do you use?

2) Which type of contract do you have (tick one):

☐

Linen rental

☐

Washing and finishing of your own sheets

☐

Other – please specify

THANK YOU FOR YOUR TIME